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ABSTRACT

"EFFECT OF TRAUMATIC STRESS ON MULTIPLE AMINERGIC SYSTEMS IN AMYGDALA AND HYPOTHALAMUS: SPECIFIC IMPAIRMENT OF 5-HT_{2A} RECEPTOR SIGNALING AND ITS PATHOPHYSIOLOGICAL ROLE IN AN ANIMAL MODEL OF POST-TRAUMATIC STRESS DISORDER"

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The amygdala and hypothalamus are central brain regions participating in stress response. This response also requires participation of multiple aminergic systems, which extensively interconnect with the amygdala and hypothalamus. Thus, dysregulation of aminergic systems, particularly the serotonergic and noradrenergic systems, is closely linked with multiple anxiety and stress disorders. The present study, utilizing a learned helplessness stress model, determined if alterations of aminergic systems in the amygdala and hypothalamus were involved in stress-induced behavioral and physiological abnormalities associated with anxiety and stress disorders.

The first part of the study investigated the electrophysiological function of serotonergic, histaminergic and noradrenergic systems in the basolateral amygdala (BLA) in control rats and associated these functions with identification of the primary aminergic receptors. The principal electrophysiological function of serotonin in the BLA was to

facilitate GABA release and the 5-HT_{2A} receptor was the primary serotonin receptor involved in this effect. The principal electrophysiological function of histamine in the BLA was to suppress excitatory synaptic transmission through presynaptic histamine H_3 receptors. In certain BLA neurons, histamine also potentiated excitatory synaptic transmission via a currently unknown mechanism. The primary function of norepinephrine in the BLA was also to suppress amygdala excitability, and the α_2 adrenoceptor was confirmed to be one of the mechanisms mediating this inhibitory effect.

In rats exposed to three-day restraint/tail shock, 5-HT_{2A} receptor-mediated serotonergic facilitation of GABA release was severely impaired, while α_2 adrenoceptor-mediated and H_3 receptor mediated suppression of BLA excitatory synaptic transmission were not significantly changed. Quantitative RT-PCR and western blot analysis further demonstrated that stress specifically decreased BLA 5-HT_{2A} receptors, while other aminergic receptors were not significantly altered. In addition, treatment with the selective 5-HT_{2A} antagonist, MDL 11,939 during stress, which is assumed to prevent amygdala 5-HT_{2A} receptor from being impaired, prevented the occurrence of enhanced acoustic startle response (ASR), a stress-induced behavioral manifestation that depends on the amygdala.

Since serotonin in the hypothalamus, by mechanisms of 5-HT_{2A},5-HT_{2C}, 5-T_{1A}, 5-HT_{1B} receptors, provides an important mechanism mediating feeding and body weight, the present study also examined whether sustained body weight loss in stressed animals is associated with dysregulation of hypothalamic serotonergic system. Stress decreased the mRNA levels of hypothalamic 5-HT_{2A} receptor and increased 5-HT_{1B} receptor mRNA levels, while 5-HT_{2C} and 5-HT_{1A} receptor mRNA levels remained unchanged.

Pretreatment with the selective 5-HT_{2A} antagonist, MDL 11,939, which is assumed to prevent hypothalamic 5-HT_{2A} receptors from being decreased by stress, dose-dependently reversed sustained body weight loss in stressed animals.

These findings indicate that BLA and hypothalamic 5-HT_{2A} receptors, but not H_3 receptor, α_2 adrenoceptor and other serotonin receptors, play a critical role in pathophysiological response to traumatic stress, and alteration of this receptor in the BLA and hypothalamus may represent an essential mechanism underlying the emergence of behavioral and physiological abnormalities resulting from stress, such as enhanced ASR and sustained body weight loss. Hence, 5-HT_{2A} receptor ligands may be potential preventive or therapeutic agents for stress-associated psychiatric disorders, especially post-traumatic stress disorder.

EFFECT OF TRAUMATIC STRESS ON MULTIPLE AMINERGIC SYSTEMS IN AMYGDALA AND HYPOTHALAMUS: SPECIFIC IMPAIRMENT OF 5-HT_{2A} RECEPTOR SIGNALING AND ITS PATHOPHYSIOLOGICAL ROLE IN AN ANIMAL MODEL OF POST-TRAUMATIC STRESS DISORDER

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List of Abbreviations

BLA, basolateral amygdala;

sIPSCs, spontaneous inhibitory postsynaptic currents;

mIPSCs, miniature inhibitory postsynaptic currents;

qRT-PCR, quantitative real time-polymerase chain reaction;

PTSD, post traumatic stress disorder;

ACSF, artificial cerebrospinal fluid;

RNA, ribonucleic acid;

cDNA, complementary deoxyribonucleic acid;

mRNA, messenger ribonucleic acid;

TTX, tetrodotoxin;

PND, postnatal day;

ASR, acoustic startle response;

EDTA, ethylenediaminetetraacetic acid;

HEPES, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid;

QX314, N-(2, 6- Dimethylphenylcarbamoylmethyl)triethylammonium chloride;

TASK, two-pore domain acid-sensitive inwardly rectifying K^+ channels

PLC, phospholipase C;

PVN, paraventricular nucleus;

CRF, corticotrophin-releasing factor;

HPA axis, hypothalamic-pituitary-adrenal axis;

TPH2, tryptophan hydroxylase 2 (TPH2);

DTT, dithiothreitol;

NMDA, *N*-methyl-D-aspartic acid;

AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid;

PV, paravalbumin;

D-APV, D-2-amino-5-phosphonovaleric acid;

EC, external capsule;

EPSPs, excitatory postsynaptic potentials;

fEPSPs, field potentials;

PPF, paired-pulse facilitation;

Ly293558, {(3*S*, 4aR, 6R, 8aR)-6-[2-(1(2) *H*-tetrazol-5-yl) ethyl]-decahydroisoquinoline-3-carboxylic acid};

SCH50911, (2s)-(+)-5, 5-dimethyl-2-morpholineacetic acid;

WCP, Whole Cell Electrophysiology Program;

EC_{50}, effective concentration of agonist to produce 50% of maximal response;

GABA, gamma aminobutyric acid;

AMPA/kainate-EPSPs, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate; (AMPA)/Kainate receptor mediated excitatory postsynaptic potentials;

NMDA-EPSPs, N-methyl-D-aspartate receptor mediated excitatory postsynaptic potentials;

TMN, tuberomammillary nucleus;

CNQX, 6-cyano-7- nitroquinoxaline-2,3-dione;

MDL 72222, Tropanyl 3,5-dichlorobenzoate;

SB 206553, 3,5-Dihydro-5-methyl-*N*-3-pyridinylbenzo[1,2-*b*:4,5-*b*']dipyrrole-1(2*H*)-carboxamide hydrochloride;

MDL 11,939, α-Phenyl-1-(2-phenylethyl)-4-piperidinemethanol;

BW 723C86, α-methyl-5-(2-thienylmethoxy)-1*H*-indole-3-ethanamine hydrochloride;

WAY 629, 1,2,3,4,8,9,10,11-octahydro[1,4]diazepino[6,5,4-jk]-carbazole hydrochloride;

PET, Positron emission tomography;

RBF, regional blood flow;

IPSP, inhibitory postsynaptic potential;

DRN, dorsal raphe nucleus;

LC, locus coeruleus;

CNS, central nervous system;

ACTH, adrenocorticotropic hormone;

PBS, phosphate-buffered saline;

ECL, enhanced chemiluminescence;

IPSPs, inhibitory postsynaptic potentials;

BNST, bed nucleus of the stria terminalis;

DMSO, Dimethyl sulfoxide.

List of Compounds and their Pharmacological properties

Compound name	Pharmacological properties
α-Methyl-5-HT	Broad 5-HT_2 receptor agonist
QX314	Sodium channel blocker
D-APV	NMDA receptor antagonist
CNQX	AMPA receptor antagonist
U73122	Phospholipase C inhibitor
TTX	Sodium channel blocker
Cyanopindolol	5-$HT_{1A/1B}$ receptor antagonist
MDL 72222	5-HT_3 receptor antagonist
Ketanserin	5-$HT_{2A/2C}$ receptor antagonist
SB 206553	5-$HT_{2B/2C}$ receptor antagonist
MDL 11,939	5-HT_{2A} receptor antagonist
BW 723C86	5-HT_{2B} receptor agonist
WAY 629	5-HT_{2C} receptor agonist
MDL 100907	5-HT_{2A} receptor antagonist
bicuculline methiodide	$GABA_A$ receptor antagonist
SCH 50911	$GABA_B$ receptor antagonist
R-alpha-methylhistamine	Histamine H_3 receptor agonist
mepyramine	Histamine H_1 receptor antagonist
cimetidine	Histamine H_2 receptor antagonist
thioperamide	Histamine H_3 receptor antagonist
triprolidine	Histamine H_1 receptor antagonist
clobenpropit	Highly potent histamine H_3 receptor antagonist
tiotidine	Histamine H_2 receptor antagonist
Ly293558	AMPA/kainate receptor antagonist
clonidine	α_2 adrenoceptor agonist
yohimbine	α_2 adrenoceptor antagonist
prozosin	$\alpha_{1/2}$ adrenoceptor antagonist

CHAPTER 1

Introduction

Overview

The ability to react to threatening events in the environment is of vital importance for an organism's survival. Any external or internal stimulus that threatens homeostasis—the normal equilibrium of body function—triggers a complex stress response. Sequentially, the voluntary nervous system, autonomic nervous system, and the neuroendocrine system are activated and mobilize response systems that supply the extra strength and energy needed to protect the body and brain from injury, prepare the organism to engage in *fight or flight*, and ultimately maintain or reestablish homeostasis.

The stress response, then, is an adaptive, healthy, and appropriate adjustment that allows the organism to ward off internal and external threats. However, if a stressor is chronic or is associated with trauma, it can lead to long-lasting pathophysiological changes in the systems that participate in this response, and ultimately result in illness. Prolonged, uninterrupted, unexpected and uncontrollable stress is an important precipitant factor in depression and chronic anxiety disorders such as post-traumatic stress disorder (PTSD).

PTSD is a debilitating anxiety disorder that develops after severe, emotionally traumatic events such as exposure to combat or terrorism, personal assault, car accidents, and natural disasters. This original traumatic event can result in a triad of symptoms-behaviors that include reexperiencing the event, avoidance/numbing, and hyperarousal/hypervigilance (Vieweg et al., 2006). Very often, this disorder is comorbid with depression and other anxiety disorders.

The Amygdala and Stress Response

Fear conditioning experiments have revealed that the amygdala complex, a group of nuclei located deep within the temporal cortex, play a central role in stressful/emotional information processing (LeDoux, 2003;LeDoux, 1994;LeDoux, 2000;Leppanen, 2006). The amygdala is believed to be an interface between events in the environment and the effector organs that control behavioral, autonomic and endocrine responses associated with emotional arousal and stress reactions (LeDoux, 1996;Nishijo et al., 1988;Knuepfer et al., 1995;Yaniv et al., 2004). This group of nuclei appears to be well situated to evaluate the emotional significance of sensory input directly from the thalamus or indirectly from the sensory cortex. Exposure to aversive stimuli (stressors) of multiple sensory systems, including olfactory, gustatory, visual and auditory modalities (Zald, 2003), can activate the amygdala. The aversive stimuli that activate the amygdala also include physical discomfort, such as pain (Tanimoto et al., 2003;Bernard et al., 1992), psychological stressors (LeDoux, 2003;LeDoux, 2000), and the disturbance of plasma homeostasis and other unpleasant interceptive sensations (Curtis et al., 2002;Brannan et al., 2001;Evans et al., 2002;Schulz et al., 1987;Reis and LeDoux, 1987). Thus, the amygdala's response to aversive stimuli appears to reflect a common, multimodal feature of amygdala coding. Of the thirteen nuclei of the amygdala, the *basolateral amygdala* (including the basolateral and lateral nuclei) and the central nucleus have been well characterized as key structures in the stress response. The basolateral amygdala are the entry nuclei that funnel and integrate aversive sensory input signals arriving from the thalamus (via internal capsule), highly processed sensory

information from cortex (via the external capsule), and the information from the hippocampus. In turn, the basolateral nuclei transduce emotional signals to the central nucleus of the amygdala, which then projects to hypothalamic and brainstem target areas that directly mediate behavioral, autonomic, and endocrine components of stress responses (LeDoux, 2003;LeDoux, 2000). For example, projections from the amygdala to the midbrain paraqueductal gray induce freezing behavior, and projections to the paraventricular nucleus of the hypothalamus induce the release of adrenal stress hormones. Therefore, the amygdala plays a crucial role in the orchestration and modulation of the organism's response to aversive, stressful events (LeDoux, 1996).

Amygdala Circuitry and Stress-Related Psychiatric Disorders

Evidence gathered from an array of scientific disciplines indicates that pathophysiological alterations in neuronal excitability in the amygdala is a characteristic feature of certain stress-associated psychiatric illnesses, including PTSD and depressive disorders (Drevets, 2000;Kalia, 2005;Manji et al., 2001;Shin et al., 2006). A variety of neuroimaging studies, for example, have consistently reported that PTSD patients show evidence of exaggerated responses in the amygdala to both traumatic reminders and general threatening stimuli.

In PTSD patients, amygdala hyperresponsivity has been reported during the presentation of personalized traumatic narratives (Rauch et al., 1996;Shin et al., 2004) and cues (Driessen et al., 2004), combat sounds (Liberzon et al., 1999;Pissiota et al., 2002) and photographs (Hendler et al., 2003;Shin et al., 1997), and trauma-related words (Protopopescu et al., 2005). Importantly, a recent PET study has shown amygdala

hyperresponsivity during the acquisition of fear conditioning in abuse survivors with PTSD (Bremner et al., 2005). In PTSD patients, the amygdala also was found to be hyperresponsive to traumatic-*unrelated* affective material, such as fearful facial expressions (Rauch et al., 2000b;Shin et al., 2005;Williams et al., 2006), as well as during neutral auditory oddball and continuous performance tasks (Bryant et al., 2005;Semple et al., 2000). Finally, amygdala activation has been shown to be positively correlated with PTSD symptom severity (Rauch et al., 1996;Shin et al., 2004;Protopopescu et al., 2005;Armony et al., 2005) and self-reported anxiety (Pissiota et al., 2002;Fredrikson and Furmark, 2003).

Depressive illness, a group of diseases that often exist comorbidly with PTSD, is another common stress-related psychiatric disorder that exhibits changes in amygdala excitability. PET scan studies have revealed multiple abnormalities of regional blood flow (RBF) and glucose metabolism in resting, unmedicated patients, with RBF and metabolism found consistently increased in the amygdala (Manji et al., 2001). Furthermore, the amygdala's RBF response to emotionally valenced stimuli are also changed in patients in some depressive subgroups (Drevets, 2003), and the elevation of amygdala RBF and metabolism is positively correlated with depression severity (Drevets, 2001). During antidepressant treatment, amygdala RBF and metabolism decreases to normative levels, compatible with evidence that chronic antidepressant administration has inhibitory effects on amygdala function in experimental animals (Drevets, 2000;Drevets, 2003;Duncan et al., 1986;Gerber, III et al., 1983;Drevets, 2000).

Taken together, a pathological increase in amygdala activity is consistently observed in certain stress-related psychiatric illnesses. This hyperactivity reflects the

hyperexcitability of amygdala circuitry. Such hyperexcitability may result from the increased glutaminergic synaptic transmission, and/or reduced inhibitory neurotransmission within the amygdala (Drevets, 2003).

The hyperexcitability of the amygdala in PTSD has been hypothesized to be due to two processes. First, the circuitry of the amygdala appears to be comprised of the low intrinsic threshold connections that are hyper-responsive to sensory input signals. Second, and perhaps additionally, amygdala hyperexcitability has been associated with dysfunctions in other neural systems, including the prefrontal cortex, and the several monoaminergic neurotransmitter systems that normally modulate stress and emotional responses via reciprocal interactions with the amygdala (Villarreal and King, 2001;Drevets, 2003). Dysregulation of the monoamine systems of the brain, especially serotonergic and noradrenergic systems, is consistently observed in these disorders. Such dysregulation in the amygdala will result in abnormal emotion/stress processing in the amygdala, and thus, may contribute to the amygdala's hyperexcitability (Drevets, 2000;Drevets, 2003;Pearlstein, 2000;Southwick et al., 1999b).

Stress-Induced Changes in Amygdala Neurocircuitry

PTSD develops in the aftermath of one or more severe, emotionally traumatic events. Stressful life events also act as a predisposing and precipitating factor in affective disorders, including depression (Strohle and Holsboer, 2003). In these stress-associated disorders, the pathological change in the amygdala and the systems that normally modulate the amygdala circuitry, including monoaminergic systems, is very likely an

acquired effect as a result of stress, other than a preexisting trait (Wurtman, 2005;van Praag, 2004b).

Indeed, the systems that participate in the stress and emotional response –so readily reshaped by experience—are among the most plastic structures in the brain; especially in response to chronic, traumatic stressful events (Sapolsky, 2003), which can induce long-lasting pathophysiological changes. For instance, repeated corticotrophin-releasing factor (CRF) infusion into the rat amygdala, which mimics chronic stress, induces a pronounced reduction in both spontaneous and evoked inhibitory postsynaptic potentials (IPSPs) in the amygdala, and result in anxiety-like symptoms in animals (Shekhar et al., 2005;Rainnie et al., 2004). This change can last for several months (Shekhar et al., 2005;Rainnie et al., 2004). In addition, chronic stress of experimental animals not only consistently causes atrophy in the hippocampus (Kim et al., 2006;McEwen, 2005;Vyas et al., 2002;Silva-Gomez et al., 2003;Magarinos et al., 1996;Rao and Raju, 1995), but also leads to an increase in dendritic arborization, dendritic elongation, and spine formation in the pyramidal neurons of the amygdala (McEwen, 2005;Vyas et al., 2004;Sapolsky, 2003;Vyas et al., 2003;Vyas et al., 2002;Vyas et al., 2006;Mitra et al., 2005). These changes appear to be irreversible after recovery from stress (Vyas et al., 2004). Finally, GABA neurotransmission is a primary regulator of neuronal excitability; chronic stress could also impair this system in the amygdala (Rodriguez Manzanares et al., 2005;Farabollini et al., 1996;Braga et al., 2004), and result in the hyperexcitability of amygdala circuits. Amygdala remodeling at a variety of sites, then, could conceivably contribute to the hyperexcitability of the amygdala as observed in the stress-associated psychiatric disorders.

As noted earlier, stressed-related psychiatric disorders have been associated with dysregulation of several monoaminergic neurotransmitter systems that interact with the amygdala. Such dysregulation can also be induced by sustained, traumatic stress in the animals. For example, changes in the serotonergic system, produced by sustained stress, substantially mimics the kinds of disturbances that are observed in stressed-related psychiatric disorders (van Praag, 2004b). Indeed, the first effective antidepressants (serotonin reuptake inhibitors) prompted the *serotonin hypothesis* as a model not only of the pathophysiology of depressive disorders, but also the neuropharmacological basis of phobias, PTSD, and generalized anxiety disorders (Hindmarch, 2002).

Serotonin, Stress, and Stress-Related Psychiatric Disorders

Substantial evidence, then, indicates that serotonin dysregulation is an important link in the pathophysiology of trauma-related symptoms. In humans, dysregulation of the serotonergic system has been closely associated with depression (Brown and Linnoila, 1990), anxiety, aggression, impulsivity (Brown and Linnoila, 1990), and suicidal behaviors (Stanley and Stanley, 1990); all symptoms that are associated with PTSD. Deficits in serotonin (5-hydroxytryptophan: 5-HT) metabolism (Maes et al., 1999;Fichtner et al., 1995;Arora et al., 1993;van Praag, 2004b) and serotonin receptor disturbances (van Praag, 2004b;van Praag, 2004a;Davis et al., 1997a) have been consistently observed in patients with PTSD and in certain subgroups of depressive patients. The alterations of serotonin 5-HT_{1A} and 5-HT_2 receptors in these disorders may be closely associated with a cognitive syndrome observed in PTSD and certain subgroups of depressive illnesses (van Praag, 2004b;van Praag, 2004a).

Figure 1

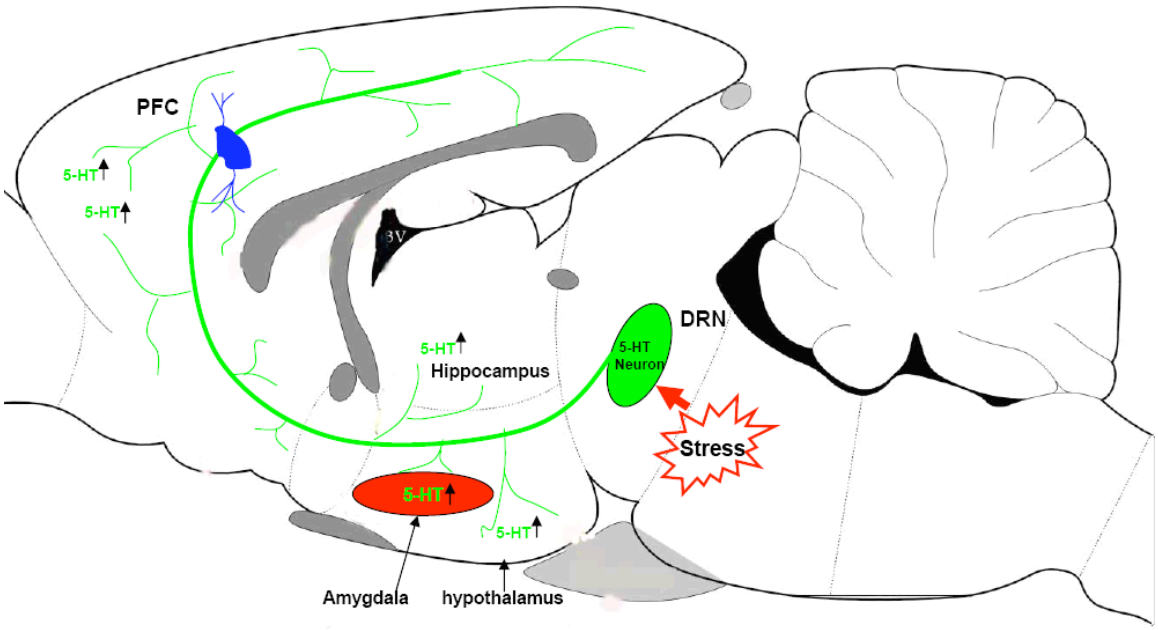

Figure 1. Inescapable stress strongly activates the dorsal raphe nucleus (DRN) and dramatically enhances 5-HT levels in its principal projecting regions, including the amygdala, prefrontal cortex, hippocampus and hypothalamus.

Such changes in the serotonin system in trauma-related disorders can be induced in experimental animals by sustained stress (van Praag, 2004b). For instance, sustained stress will lead to a decrease in 5-HT turnover in rats (van Praag, 2004b). Chronic stress also alters 5-HT_{1A} and 5-HT_2 receptor expression and signaling in the brain regions that participate in stress and emotion response in rodents, including the hippocampus (van Riel et al., 2003;Matsumoto et al., 2005;Dwivedi et al., 2005), prefrontal cortex (Harvey et al., 2003;Dwivedi et al., 2005), amygdala (Wu et al., 1999), and hypothalamus (Dwivedi et al., 2005;Wu et al., 1999).

Over the past 20 years, many studies have used experimental animals, in an inescapable stress test condition, to understand the role of the serotonergic system in the pathogenesis of depression and anxiety-like symptoms (Maier and Watkins, 2005;Minor and Hunter, 2002;Amat et al., 2005;Robbins, 2005). These studies demonstrated that inescapable stress strongly activates the dorsal raphe nucleus (DRN), which dramatically increases serotonin levels in its principal projection sites, including the amygdala, prefrontal cortex, hippocampus and hypothalamus (see Fig.1). The activation and subsequent sensitization of DRN 5-HTergic neurons appears to be responsible for the behavioral changes associated with depression and anxiety disorders (Maier and Watkins, 2005;Minor and Hunter, 2002;Amat et al., 2005;Robbins, 2005). Since the amygdala is the brain region with the highest serotonergic input from the DRN (Jacobs and Azmitia, 1992;Tork, 1990), the activation and sensitization of DRN 5-HTergic neurons following inescapable stress, can induce heightened 5-HT levels in the amygdala for a long period of time (Amat et al., 1998;Maier and Watkins, 2005;Minor and Hunter, 2002). Long-term

exposure of the amygdala neuron to elevated levels of 5-HT, then, may play a critical role in the development of aversive consequences of stress (Minor and Hunter, 2002).

Physiologically, serotonergic transmission in the amygdala appears to determine the neuronal excitability of the amygdala in the generation and regulation of the stress/emotional behaviors. Thus, genetic variations of the human serotonin transporter and serotonin synthesizing enzyme (TPH2), which determine their enzymatic efficiency and subsequently synaptic serotonin availability, can affect amygdala neuronal activity in response to emotional stimuli and predispose the individual to multiple anxiety and stress disorders (Brown et al., 2005;Canli et al., 2005;Hariri et al., 2002;Hariri et al., 2005). Since serotonergic neurotransmission plays such a crucial role in determining neuronal excitability of the amygdala, modification of this system in the amygdala, as a consequence of stress, could lead to a threshold change of the amygdala's response to sensory input, and result in abnormal emotional/stressful information processing. Thus, to determine the 5-HT functional change in the amygdala as a function of stress is of great interest for understanding the development of aversive outcomes of stress. However, such functional changes have never before been examined.

Although serotonergic modulation of the amygdala's excitability is so important for emotional/stressful information processing, the exact functional role of serotonin in this modulation remains inconclusive. Several studies suggest that serotonin modulates amygdala excitability by exciting interneurons and increasing GABAergic synaptic transmission in the basolateral amygdala (BLA), especially at relatively low concentrations (Rainnie, 1999;Stutzmann et al., 1998;Stutzmann and LeDoux, 1999;Stein et al., 2000). The receptors involved in this effect may be the 5-HT_2 receptor (Rainnie,

1999;Stein et al., 2000) and/or the 5-HT_3 receptor (Rainnie, 1999;Stein et al., 2000;Koyama et al., 2002;Morales and Bloom, 1997). At high concentrations, serotonin appears to suppress excitatory synaptic transmission in the BLA (Cheng et al., 1998;Rainnie, 1999). 5-HT_{1A} receptors may be involved in this effect (Cheng et al., 1998), although this claim was questioned by another study (Rainnie, 1999). Taken together, serotonin appears to exert a strong inhibitory effect on amygdala excitability, but the receptor subtypes involved in this effect are not clear.

Other Monoamines, Stress, and Stress-Related Psychiatric Disorders

Research directed at understanding the consequence of traumatic stress and the pathogenesis of stress-associated psychiatric disorders has not only focused on the serotonergic system, but also other aminergic systems. In particular, dysregulation of the noradrenergic system, like the serotonergic system, has also long been closely associated with multiple anxiety and stress disorders, including PTSD and depressive illnesses (Leonard, 2001;Manji et al., 2001;Southwick et al., 1999a).

The noradrenergic system originates in the locus coeruleus (LC), and provides the majority of *noradrenergic* innervation to the amygdala, prefrontal cortex, and hippocampus. Release of norepinephrine in these corticolimbic structures during stressful situations is a critical limb of the stress/fear response. Stress dramatically increases norepinephrine in the amygdala (Galvez et al., 1996;Quirarte et al., 1998;Tanaka et al., 1991;Torda et al., 1985), and increased noradrenergic neurotransmission within the amygdala during stress is critically involved in enhanced encoding of emotional memories, sensitization, and fear conditioning (see Fig.2).

Figure 2

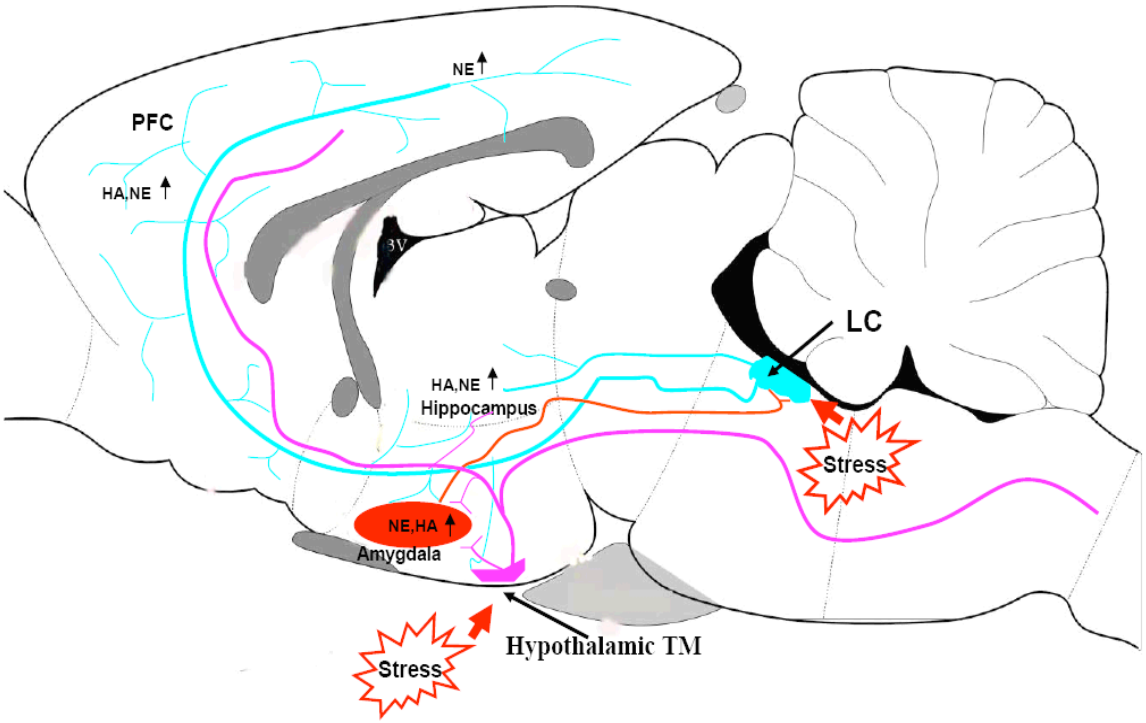

Figure 2. The noradrenergic (green) and histaminergic (pink) innervation of the forebrain and their responses to stress. The majority of norepinephrine (NE) in the forebrain comes from the locus coeruleus (LC). Stress can activate the LC neurons and increase the NE levels in the amygdala, hippocampus, prefrontal cortex and hypothalamus (Berridge and Waterhouse, 2003). Increased noradrenergic neurotransmission within the amygdala during stress is critically involved in enhanced encoding of emotional memories, sensitization, and fear conditioning. Histamine-releasing neurons are located exclusively in the tuberomammillary nucleus (TM) of the hypothalamus, and project to practically all brain regions, with ventral areas (hypothalamus, basal forebrain, amygdala) receiving a particularly strong innervation. Histamine (HA) release is enhanced under extreme conditions such as dehydration or hypoglycemia or by a variety of stressors (Brown et al., 2001).

In an attempt to understand the pathogenesis of depressive illness and anxiety disorders, animal studies have shown that chronic stress could induce changes in the noradrenergic system, very similar to what has been observed in certain subgroups of depressive illness and PTSD (Leonard, 1997b;Leonard, 1997a;Leonard, 2001;Brunello et al., 2002;Braga et al., 2004). Among these changes, alterations in α_1 and α_2 adrenoceptor levels have been repeatedly reported in the amygdala and cortex (Leonard, 1997a;De Paermentier et al., 1997;Braga et al., 2004). Chronic stress downregulates α_2 adrenoceptors in the LC (Flugge, 1996;Simson and Weiss, 1988), amygdala (Coplan et al., 1992;Nukina et al., 1987), hippocampus (Nukina et al., 1987), and prefrontal cortex (Coplan et al., 1992;Flugge, 1996). Since the α_2 adrenoceptor primarily serves as an autoreceptor for controlling locus coeruleus firing and norepinephrine release, subsensitivity of this receptor could result in increased noradrenergic activity, which may be responsible for hyperarousal in PTSD including hypervigilance, exaggerated startle, anger, and insomnia (Lemieux and Coe, 1995;Yehuda et al., 1992;Southwick et al., 1993), and intrusive traumatic memories (Southwick et al., 1997).

While histaminergic neurotransmission has received much less attention than the other aminergic systems, it is likely to be of comparable importance. This system may also be implicated in stress-related psychiatric disorders, especially in depressive illnesses (Kano et al., 2004). Actually, many antidepressants, supposedly acting on serotonergic and noradrenergic systems, also interact with the histaminergic system (Brunello et al., 2002). This system, like the serotonergic and noradrenergic system, prominently innervates stress/emotional response systems, including the hypothalamus, basal forebrain and amygdala in rats (Panula et al., 1989;Brown et al., 2001). Through its

innervation of these brain regions, then, histaminergic neurons play an important role in modulating stress and emotional responses (Brown et al., 2001)(see Fig.2). Although a change of this system has not been extensively reported in stress-related disorders, it is worthy to investigate the effect of chronic, traumatic stress on histaminergic neurotransmission, not only because this system may serve as a negative control, but also it may provide a possible new research direction for the study of psychiatric disorders.

An Animal Model for Studying Stress-Related Psychiatric Disorders

The development of an animal model is critical for the study of therapeutic and prophylactic treatments of stress-associated psychiatric disorders, such as PTSD. Since it is generally recognized that the emotional and health-related consequences of aversive experiences are much worse when the organism has no control over the aversive event, chronic uncontrollable stress (learned helplessness) has been adopted as an animal model of depressive illness (Drevets, 2003;Maier and Watkins, 2005;Minor and Hunter, 2002). Indeed, chronic, uncontrollable stress causes behavioral, endocrine, immune and neurotransmitter changes in the animals that are qualitatively similar to those observed in depressive illness associated with anxiety symptoms (Leonard, 2001). Since PTSD, to some extent, overlaps with depressive illness in terms of etiology and certain features, the animal model of PTSD modified from the learned helplessness paradigm has been shown to mimic to a substantial extent PTSD in terms of certain physiological parameters (Servatius et al., 1995;Garrick et al., 2001;Braga et al., 2004). This lab and other labs have successfully established and tested the inescapable tail-shock model of stress in rats and verified that the behavioral and neurobiological alterations induced by this rat model

are similar to those found in PTSD patients (Servatius et al., 1995;Garrick et al., 2001) (Table 1)

Table 1. Comparison of Symptoms of PTSD in Humans to Stress-induced Dysfunctions in Rats.

PTSD in Humans	Inescapable tail-shock model of stress in rats
Weight loss (Sutker et al., 1990;Myers et al., 2005)	Suppressed feeding and body weight loss (Hu et al., 2000;Harris et al., 2002)
Difficulty falling or staying asleep, nightmares (Maher et al., 2006;Maher et al., 2006)	Altered sleep patterns (Adrien et al., 1991)
Psychomotor numbness (Epstein et al., 1998;Lopez-Ibor, 2002)	Persistent behavioral abnormalities i.e. suppressed open-field activity, longer hanging wire latencies (Minor et al., 1984;Pare, 1994)
Poor concentration; memory deficits (Bremner et al., 2004;Jelinek et al., 2006;Isaac et al., 2006;Green, 2003)	Deficits in escape/avoidance learning and learning of an appetitive task (Maier, 2001)
Hypervigilance and/or exaggerated startle response (Orr et al., 2002;Orr and Roth, 2000;Pitman et al., 1999)	Exaggerated startle (Garrick et al., 2001;Servatius et al., 1995)
Hyperresponsiveness of the noradrenergic system (Orr and Roth, 2000;Maes et al., 1999)	Hyperresponsiveness of the noradrenergic system (Simson and Weiss, 1988)

Using this animal model, we examined whether the serotonergic system in the amygdala is modified after exposure to traumatic stress. Alterations in the serotonergic system in the amygdala as a function of stress will be evaluated by electrophysiological, molecular, and behavioral approaches. If stress does induce an alteration in this system, the relationship between this functional change and physiological, behavioral outcomes of such stress will be examined utilizing weight gain and acoustic startle as measurable parameters. Also, the functional change of the noradrenergic and histaminergic system in the amygdala will also be examined in this animal model not only to determine the specificity of stress-induced change of the serotonergic system, but to try and explore the possible role of the noradrenergic and histaminergic systems in the pathophysiology of

stress. Since the electrophysiological function of histamine and serotonin in the amygdala is primarily unknown, first efforts investigate the physiology of these two systems in the amygdala.

CHAPTER 2

Traumatic Stress Impairs 5-HT_{2A} Receptor-Mediated GABA release in Basolateral Amygdala: 5-HT_{2A} receptor blockade Prevents Stress-Induced Enhanced Startle Response

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ABSTRACT

The occurrence of anxiety and stress disorders has been closely associated with alterations of amygdala GABAergic synaptic transmission. In these disorders, dysregulation of the serotonergic system, a very important modulator of amygdala GABAergic system, is also well recognized. The present study, utilizing a learned helplessness stress rat model, was designed to determine if behavioral and physiological abnormalities resulting from stress are associated with alterations of serotonergic modulation of amygdala GABAergic system. In control rats, administration of 5-HT or α-Methyl-5-HT, a 5-HT_2 receptor agonist, to basolateral amygdala (BLA) slices dramatically enhanced frequency and amplitude of spontaneous inhibitory postsynaptic currents (sIPSCs). This effect was blocked by the selective 5-HT_{2A} receptor antagonists, MDL11,939 and MDL 100,907, while the selective 5-HT_{2B} receptor agonist, BW 723C86, and the selective 5-HT_{2C} receptor agonist, WAY 629, were without effect on sIPSCs. Double immunofluorescence labeling demonstrated that the 5-HT_{2A} receptor is primarily localized to parvalbumin-containing BLA interneurons. These observations indicated that 5-HT_{2A} receptors mediated serotonergic facilitation of BLA GABA release. In stressed rats, 5-HT_{2A} receptor-mediated facilitory effects on sIPSCs were severely impaired. Quantitative RT-PCR and western blot analysis showed that stress downregulated BLA 5-HT_{2A} receptors. Treatment with the selective 5-HT_{2A} antagonist, MDL 11,939 before stress or immediately after stress, which is assumed to prevent amygdala 5-HT_{2A} receptor from being downregulated, prevent the occurrence of enhanced acoustic startle response (ASR), a stress-induced behavioral manifestation that depends on the amygdala. These findings suggest that impaired BLA 5-HT_{2A} receptor signaling is closely associated with

stress-enhanced ASR, and 5-HT_{2A} receptor antagonists may be effective prophylactic or therapeutic agents for stress-associated psychiatric disorders, such as PTSD.

INTRODUCTION

The ability to react to threatening events in the environment is of vital importance for an organism's survival. However, it is well recognized that the extreme and/or continued stimulation of the circuits and systems that respond to threat or danger may induce long-lasting pathophysiological alterations in these systems and result in or accelerate the pathogenesis of psychiatric disorders (McEwen, 2003;Shekhar et al., 2005;Vermetten and Bremner, 2002;McEwen, 2005). In the brain, a central structure that participates in the stress/danger response is the amygdala, a group of nuclei located deep within the temporal cortex (LeDoux, 1996;LeDoux, 2003;LeDoux, 1994). The basolateral nuclei of the amygdala (BLA), via reciprocal connections with the cerebral cortex, the thalamus, and other subcortical structures, receives input from different sensory modalities and determines the emotional significance of external events (LeDoux, 1996;Nishijo et al., 1988;Knuepfer et al., 1995;Yaniv et al., 2004). Via the interconnection between the BLA and central nucleus, and its efferent pathways to the hypothalamus and brainstem, the amygdala transduces emotional signals to the hypothalamic-pituitary-adrenal (HPA) axis, autonomic and motor centers that initiate different components of the stress response (LeDoux, 2003;LeDoux, 2000). Since the amygdala occupies such a central role in the stress response, understanding the impact of intensive and/or chronic stress on the amygdala's physiology is critical in understanding the pathophysiology of stress, and may aid in unraveling the pathogenesis of stress-associated psychiatric disorders such as PTSD and depressive disorders.

Neuronal circuitries in the BLA are inter-connected extensively with GABAergic terminals (Nitecka and Ben-Ari, 1987;Washburn and Moises, 1992). Activity of

amygdaloid projection neurons is under strong inhibitory control by GABAergic neurotransmission (Royer et al., 1999;Szinyei et al., 2000). Manipulating this system within the BLA has a critical influence on behavioral, emotional sequelae resulting from stress (Jasnow and Huhman, 2001;Kim et al., 2005;Sanders and Shekhar, 1995;Van Nobelen and Kokkinidis, 2006). Thus, the changes in the activation of BLA GABAergic system have been closely associated with the occurrence of stress-associated psychiatric syndromes. For instance, repeated corticotrophin-releasing factor (CRF) infusion into the rat amygdala, which mimics chronic stress, induces a pronounced reduction in both spontaneous and evoked inhibitory postsynaptic potentials (IPSPs) in the amygdala that result in anxiety-like symptoms in animals (Rainnie et al., 2004). Intensive stress attenuates inhibitory GABAergic control in the BLA (Braga et al., 2004;Rodriguez Manzanares et al., 2005), which appears to be responsible for behavioral impairments resulting from stress (Minor and Hunter, 2002). In addition, many drugs for mood and anxiety disorders achieves their therapeutic effects at least partially by modifying the GABAergic system in the amygdala (Gerber, III et al., 1983;Millan, 2003;Schallek and Schlosser, 1979;Taylor, 2003). Hence, stress-induced alteration of GABAergic neurotransmission in the amygdala, may be an important pathophysiological mechanism of anxiety and stress disorders, including PTSD and depressive illnesses.

The dorsal raphe (DRN) serotonergic system provides the majority of serotonin for the forebrain (Molliver, 1987;Jacobs and Azmitia, 1992;Hensler, 2006). This system is a very important modulator of the GABAergic system in the amygdala. The BLA is the brain region with the heaviest innervation of serotonergic terminals from the DRN (Jacobs and Azmitia, 1992;Tork, 1990). Serotonin levels could be strongly enhanced in

the basolateral amygdala by stressful/emotional experience, and this enhancement remains for a long period of time (Amat et al., 1998;Maier and Watkins, 2005;Minor and Hunter, 2002). The primary action of this enhanced serotonergic neurotransmission is to reduce amygdala excitability by increasing GABAergic synaptic transmission in the BLA (Rainnie, 1999;Stutzmann et al., 1998;Stutzmann and LeDoux, 1999). Such serotonergic modulation in the amygdala appears to be of great importance in normal emotional/stressful signal processing (Canli et al., 2005;Hariri et al., 2002), and dysregulation in the 5-HTergic system have been associated with multiple anxiety and stress disorders (Southwick et al., 1997;Manji et al., 2001;Eison and Eison, 1994). Hence, malfunctioning of serotonergic modulation in the amygdala may play a critical role in the occurrence of symptoms associated with anxiety and stress disorders. However, the association between the alteration of serotonergic modulation in the amygdala and occurrence of physiological and behavioral abnormalities associated with anxiety and stress disorders has not been previously examined.

Thus, the present study investigated whether serotonergic modulation of GABAergic transmission in the BLA is altered by exposure to restraint-tail shock, a stress protocol which could stably induce abnormalities associated with stress-related disorders. Furthermore, if alterations do occur, this study also determined if serotonergic alterations in the amygdala is associated with the occurrence of stress-induced behavioral abnormalities that depend on the amygdala, such as enhanced ASR (Servatius et al., 1995;Garrick et al., 2001). Since the receptor subtypes involved in serotonergic modulation of GABA release in the amygdala are not entirely clear, we first determined which serotonin receptors are associated with serotonergic modulation of GABA release.

METHODS

Stress Protocol

All animal experiments were performed in accordance with our institutional guidelines after obtaining the approval of the Institutional Animal Care and Use Committee (IACUC). Male, Sprague–Dawley rat pups were received with their mother at postnatal day (PND) 17 and housed in a climate controlled environment on a 12 h light/dark cycle (lights on at 0700). On PND 21, the rats were weaned, assigned numbers, and randomly divided into control and stressed groups. They were housed individually, with food and water supplied *ad libitum*. The 'stressed group' was exposed to stress on PND 22, 23, and 24. The rats were sacrificed and brain slices were prepared on PND 24 and 25. The experiments were performed in a blind manner. The investigators did not know whether they used a control or a stressed rat until the data were analyzed.

Stress exposure consisted of a 2-h per day session of immobilization and tail-shocks, for 3 consecutives days. The animals were stressed in the morning (between 0800 and 1200). They were restrained in a plexiglas tube, and 40 electric shocks (1 mA, 3 s duration) were applied at varying intervals (140–180 s). This stress protocol was adapted from the 'learned helplessness' paradigm in which animals undergo an aversive experience under conditions in which they can not perform any adaptive response (Seligman and Maier, 1967;Seligman and Beagley, 1975). The rats were stressed for 3 consecutive days because it has been previously demonstrated that repeated stress sessions for 3 days is more effective than a single stress session in producing physiological and behavioral abnormalities, such as elevations in the basal plasma corticosterone levels, exaggerated acoustic startle responses (ASR), and reduced body

weight (Ottenweller et al., 1989;Servatius et al., 1995). More stress sessions, beyond the 3 days, do not appear to produce greater physiological and behavioral changes (Ottenweller et al., 1989;Servatius et al., 1995).

Slice Preparation

The amygdala slice preparation has been described previously (Li et al., 2001). Briefly, the rats were anesthetized with halothane and then decapitated. The brain was rapidly removed and placed in an ice-cold artificial cerebrospinal fluid (ACSF) composed of (in mM) 125 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, and 11 glucose, bubbled with 95% O_2/5% CO_2. A block containing the amygdala region was isolated by rostral and caudal coronal cuts, and coronal slices, 400 μm thick, were cut using a Vibratome (series 1000, Technical Products International, St Louis, Missouri). Slices were kept in a holding chamber containing oxygenated ACSF at room temperature, and experiments started ≥1 h after slice preparation.

Electrophysiology

For whole-cell recordings, slices were transferred to a submersion-type recording chamber where they were continuously perfused with oxygenated ACSF at a rate of 2 ml/min. All experiments were carried out at room temperature. Tight-seal (≥1GΩ) whole-cell recordings were obtained from the cell body of neurons in the BLA region. Patch electrodes were fabricated from borosilicate glass and had a resistance of 1.5–5.0MΩ when filled with a solution containing (in mM) Cs-gluconate, 135; $MgCl_2$, 10; $CaCl_2$, 0.1;

EGTA, 1; HEPES, 10; QX-314, 20; NaATP, 2; Na_3GTP, 0.2 and Lucifer yellow, 0.4% (pH 7.3, 285–290 mOsm). Neurons were visualized with an upright microscope (Olympus BX51WI) using Nomarski-type differential interference optics through a 40x water immersion objective. Neurons with a pyramidal appearance were selected for recordings. During whole-cell recordings, neurons were filled passively with 0.4% Lucifer yellow (Molecular Probes, Eugene, Oregon) for *post hoc* morphological identification, as described previously (Braga et al., 2003). The fluorescence image of the dye-filled neurons was captured by a Leica DM RXA fluorescence microscope equipped with an SPOT2 digital camera and a laser scanning confocal microscope (Bio RAD, MRC-600). Neurons were voltage clamped using a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA). Inhibitory postsynaptic currents (IPSCs) were pharmacologically isolated and recorded at a holding potential of -70 mV. Access resistance (8–26MΩ) was regularly monitored during recordings, and cells were rejected if it changed by more than 15% during the experiment. The signals were filtered at 2 kHz, digitized (Digidata 1322A, Axon Instruments, Inc.), and stored on a computer using pCLAMP8 software (Axon Instruments, Inc.). The peak amplitude, 10–90% rise time, and the decay time constant of IPSCs were analyzed off-line using pCLAMP8 software (Axon Instruments) and the Mini Analysis Program (Synaptosoft, Inc., Leonia, NJ).

Quantitative real-time PCR

The amygdala complex was dissected and kept frozen. Frozen tissues of 30 mg were homogenized, and total RNA was extracted using the RNeasy kit (Qiagen, Germany) following the manufacturer's protocol. One microgram (1 µg) of total RNA

was reverse transcribed into first-strand cDNA using the RETROscript reverse transcriptase kit and oligo dT primers (Ambion Inc., Austin, TX) according to the manufacturer's protocols. One microliter (1 µl) of cDNA from the RT-reaction was used as the template for quantitative real-time PCR reaction with a final PCR reaction volume of 25 µl, with the 5' and 3' gene specific PCR primer concentrations at 10 pM each. PCR primers were designed using Primer3 software (Whitehead Institute, MIT, MA) according to the coding sequences of each gene (GenBank#: 5-HT_{2A} receptor, #X13971; 5-HT_{2C} receptor, # NM_012765) (Table 1). Quantification of mRNA expression was performed (in triplicate) using the SYBR Green SuperMix (BioRad, CA) and a 2-step PCR reaction procedure, performed on a MyiQ Single Color Real-Time PCR Detection System (BioRad, CA). In brief, after the initial denaturation at 95^{o}C for 3 min, 45 cycles of primer annealing and elongation were conducted at 60^{o}C for 45 seconds, followed by denaturation at 95^{o}C for 10 s. Fluorescent emission data were captured, and mRNA levels were quantified using the threshold cycle value (Ct). To compensate for variations in input RNA amounts and efficiency of reverse transcription, data for 5-HT_{2A} receptor and 5-HT_{2C} receptor mRNA for each sample were normalized by reference to the data obtained for the house keeping β-actin gene (GenBank accession #. BC063166) determined from the same sample. Fold change in mRNA expression was calculated using the equation: fold change=$2^{-\Delta\Delta Ct,}$ where ΔCt=target gene Ct-house keeping gene (β-actin) Ct, and ΔΔCt is ΔCt control - ΔCt stress (or fold change) =$2^{\wedge(\Delta CT\ control\ -\ \Delta CT\ stress)}$. The mean and S.E.M. were calculated from three replicate amplifications. Each RT-PCR assay was repeated twice.

Differences between the stress and control groups and between the brain regions

were examined for statistical significance using one-way ANOVA (with stress or brain region as the main factors) analysis followed by post-hoc Fisher's test. A difference with *P*-value less than 0.05 was considered statistically significant.

Table 1. $5HT_2$ receptor *PCR primer sequences*

Gene	Sense Primer (5'-3')	Antisense Primer (5'-3')
5HT2A	ATACCAGCATTGGCCTACAAGT	TAACCATGGAGCAGTCATCAAC
5HT2C	AGCCCAGACCATTTCTAATGAA	TGAGAGTAGTCTGGTTGCAGGA

Western blot analysis

Immunolabeling of 5-HT_{2A} receptors was determined by the procedure described earlier (Zhang et al., 2003). Tissues were homogenized in homogenizing buffer containing 5 mM Tris–HCl (pH 7.4), 0.1% EDTA, 2 mM leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 1.45 mM pepstatin, 0.2 unit/ml aprotinin, and 2 mM DTT. The homogenate was centrifuged at 980×g for 10 min at 4 ^{O}C. The supernatant was recentrifuged at 65,000×g for 15 min at 4 ^{0}C. The resulting pellet was resuspended in homogenizing buffer. Equal volumes of protein samples (20 µl containing 25 µg protein) were loaded onto 7.5% (w/v) polyacrylamide gel and subsequently transferred to an ECL nitrocellulose membrane (Amersham). The blots were incubated overnight with primary 5-HT_{2A} receptor antibody (Pharmingen, CA, USA) at a dilution of 1:500, followed by horseradish-peroxidase-linked secondary antibody (anti-mouse IgG; 1:500) for 4 h at room temperature. The membranes were exposed to enhanced chemiluminescence (ECL) film. The specificity of the antibody was checked by competition with a 5-HT_{2A} receptor fusion protein. To normalize the data, β-actin was measured in the same immunoblot using anti-β-actin as the monoclonal primary antibody (1:5000 for 2 h) and anti-mouse

IgG (1:5000 for 2 h) as the secondary antibody. The optical density of each 5-HT_{2A} receptor band was corrected by the optical density of the corresponding β-actin band.

Immunofluorescence labeling

Five rats (50-75 g) were deeply anesthetized with halothane and transcardially perfused with 100 ml of 37 °C 0.9% sodium chloride solution followed by 200 ml of freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Brains were removed and post-fixed in 4% paraformaldehyde overnight. Serial 40 μm coronal sections were cut from each brain with a Vibratome (series 1000, Technical Products International, St Louis, Missouri). The sections with the amygdala, corresponding to a rat brain atlas, were carefully selected and kept in 0.01M PBS for double immunofluorescence labeling.

The sections were incubated with the primary antibody rabbit anti-5-HT_{2A} receptor (1:100, ImmunoStar, Inc.) alone or in a cocktail of this antibody and mouse anti-parvalbumin (1:2000, Sigma Chemical Co.) overnight at 4°C, rinsed in three changes of PBS (10 min each), and then incubated with Alexa-488-labeled goat anti-mouse IgG (1:500; Molecular Probes, Eugene, OR, USA; 2.5 h) and Alexa-568-labeled goat anti-rabbit IgG (1:1000, Molecular Probes, Eugene, OR, USA; 2.5 h) at room temperature. Sections were then rinsed in three changes of PBS (10 min each) and mounted on glass slides using Vectashield mounting medium (Vector Laboratories). Sections were examined with a Bio-Rad MRC-600 confocal laser scanning microscope equipped with an argon–krypton laser attached to a Nikon Optiphot fluorescence microscope. Fluorescence of Alexa 488 (green) and Alexa 568 (red) dyes was analyzed using filter

configurations for sequential excitation/imaging via 488 nm and 568 nm channels. Digital images were adjusted for brightness and contrast using Photoshop software.

Acoustic startle measurement.

A total of 40 male Sprague–Dawley rats weighing approximately 100-150 g (Taconic Farms, Germantown, NY, USA) were used in an acoustic startle measurement study. Animals were housed in groups of two per cage with free access to food and water, and were maintained on a reverse light/dark cycle (light on 18:00) at 22 °C. To minimize the effects of handling and stress on drug testing, animals were allowed to acclimate to the startle chamber (see below) for 3 days before experiments.

The animals were equally assigned to five groups based on their body weight and baseline startle response. The five groups include a control group; a stress + vehicle group, in which animals received 0.01N HCl as vehicle before or after they underwent the stress protocol as mentioned before, each day for three consecutive days; a pre-stress low-dose group, in which animals received 0.5 mg/kg 5-HT_{2A} receptor antagonist MDL 11, 939 30 minutes before stress each day for three consecutive days; a pre-stress high-dose group, in which animals received 1.5 mg/kg MDL 11,939 30 minutes before stress each day for three consecutive days; and a post-stress group, in which animals received 1.5 mg/kg MDL 11,939 immediately after stress each day for three consecutive days. The drugs or vehicle were delivered by intraperitoneal injection (1ml latex free syringe w/ 27G½ needle, B-D, Franklin Lakes, NJ).

The acoustic startle reflex test was conducted with a Startle Response Acoustic Test System (Coulbourn Instruments, Columbus, Ohio, USA). The system consists of

four weight sensitive platforms in a sound-attenuated chamber, though only one platform was used at a time. A subject's movements in response to stimuli were transduced into analog signals by a piezoelectric unit attached to the platforms. These signals were then digitized and stored by a computer. All acoustic stimuli were given from a loudspeaker mounted 24 cm above the test cage. During testing, rats were individually placed in the animal holder (E05-15, Coulbourn Instruments, Columbus, Ohio, USA), which were then placed on weight-sensitive platforms. A ventilating fan built into the chamber provides the background noise. Following a 3-min acclimation period, animals were exposed to six types of stimulus trials: both 100 and 110 dB alone, both with pre-pulse (84dB), pre-pulse alone and no stimulus. Each trial type is presented eight times. Trial types are presented in a random order to avoid order effects and habituation. Inter-trial intervals range randomly from 15 to 25s. All animals were tested on Day 10 and Day 21 following the final stress protocol. Animals were weighed throughout the experiments, both as a physiological measure and as a metric for balancing the groups.

Each animal's responses are averaged within trial type. Trials during which no stimuli are presented are used to control for normal subject movements on the platform. Amplitudes to each trial type are derived by subtracting grams (g) of platform displacement on the no-stimulus trials (i.e., the body weight of each subject) from g of platform displacement in response to specific stimuli. The remainder from this calculation represented the amount of platform displacement related to the stimulus (e.g., 100 dB, 100 dB with pre-pulse, 110 dB, 110 dB with pre-pulse). For each test day, ANOVAs for repeated measures were performed on startle amplitudes with factors of stress status and drug dosage. The data were represented as mean ± S.E.M.

Drugs

The compounds from Tocris (Tocris Cookson, Ballwin, Missouri) include: D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5); 6-cyano-7- nitroquinoxaline-2,3-dione (CNQX); 4-[3-[*tert*-Butylamino]-2-hydroxypropoxy]-1*H*-indole-2-carbonitrile hemifumarate (Cyanopindolol hemifumarate); Tropanyl 3,5-dichlorobenzoate (MDL 72222); 2-Methyl-5-hydroxytryptamine hydrochloride; 3-[2-[4-(4-Fluorobenzoyl)-1-piperidinyl]ethyl]-2,4[1*H*,3*H*]-quinazolinedione tartrate (Ketanserin tartrate); 3,5-Dihydro-5-methyl-*N*-3-pyridinylbenzo[1,2-*b*:4,5-*b*']dipyrrole-1(2*H*)-carboxamide hydrochloride (SB 206553 hydrochloride); α-Phenyl-1-(2-phenylethyl)-4-piperidinemethanol (MDL 11, 939); α-methyl-5-(2-thienylmethoxy)-1*H*-indole-3-ethanamine hydrochloride (BW 723C86 hydrochloride); α-Methyl-5-hydroxytryptamine maleate; 1,2,3,4,8,9,10,11-octahydro[1,4]diazepino[6,5,4-jk]-carbazole hydrochloride (WAY 629); N-(2,6-Dimethylphenylcarbamoylmethyl) triethylammonium chloride (QX 314 chloride); 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122). MDL 100907 is kindly donated by Kenner C. Rice (NIDDK, National Institutes of Health). The following compounds are from Sigma: bicuculline methiodide, a $GABA_A$ receptor antagonist; tetrodotoxin (TTX), a sodium channel blocker.

RESULTS

1. Serotonin facilitated GABA release in the BLA *in vitro*.

As previous studies have indicated, serotonin can facilitate GABA release in the basolateral amygdala, and this effect may involve the 5-HT_2 receptor. To investigate whether serotonin modulates GABAergic transmission in the BLA by a 5-HT_2 receptor mechanism, we first examined the effects of serotonin on action-potential dependent sIPSCs recorded from BLA pyramidal neurons in control rats. The sIPSCs were recorded at a holding potential of -70 mV, and in the presence of D-AP5 (50 μM), CNQX (10 μM), MDL 72222 (20 μM), and Cyanopindolol (20 μM) to block NMDA, AMPA/kainate, and 5-HT_1 and 5-HT_3 receptors, respectively. In control rats, the mean frequency of sIPSCs recorded from the soma of BLA pyramidal neurons was 2.8 ± 1.5 Hz (n=21). Bath application of bicuculline (10 μM) eliminated sIPSCs, confirming that they were mediated by $GABA_A$ receptors. Serotonin produced a dose-dependent enhancement in the frequency and amplitude of sIPSCs. After application of 50 μM serotonin, the mean frequency of sIPSCs was increased to 1190.0 ± 95.1% of the baseline values (n=8, $p<0.01$; Figure 1A, B, D). The amplitude of sIPSCs was increased to 182.6 ± 16.1% of the baseline values (n=8, $p<0.05$; Figure 1A, D). These effects persisted throughout the application of serotonin and were completely reversed after removal of the agonist. The effects of serotonin were not accompanied by any significant change in the rise time or decay time constant of sIPSCs. The effect could be mimicked by the broad 5-HT_2 receptor agonist α-Methyl-5-HT. α-Methyl-5-HT, at concentrations of 2, 15, and 100 μM,

increased the frequency and amplitude of sIPSCs in a dose-dependent manner (Figure 2C). The concentration of 15 μM appears to be close to the EC_{50}, and therefore it was used in subsequent experiments. Application of 15 μM α-Methyl-5-HT increased frequency of sIPSCs to 612.8±70.9 % of the baseline values (n=16, p<.01; Figure 2A, B, C). The amplitude of sIPSCs was increased to 172.3±16.6 % of the baseline values (n=16, p<0.05).

2. The facilitatory effect of serotonin was mediated by 5-HT_{2A} receptors in the BLA.

To identify the subtypes of the 5-HT_2 receptor involved in the effects of serotonin and α-Methyl-5-HT on sIPSCs in control rats, we first pretreated the slices with the 5-$HT_{2A/2C}$ receptor antagonist Ketanserin (10 μM) for 30 minutes. In the presence of Ketanserin, applied α-Methyl-5-HT (15 μM) no longer induced any change in the frequency and amplitude of sIPSCs, indicating that the 5-$HT_{2A/2C}$ receptor may be responsible for the facilitory effect. Thus, the frequency of sIPSCs before and after application of α-Methyl-5-HT in the presence of Ketanserin is 2.84 ±1.1 and 2.69±0.9 Hz, respectively (n=11, p>0.05, Figure 3E).

The selective 5-HT_{2A} receptor antagonists, MDL 11,939 and MDL 100,907, were used to examine whether the effect is mediated by the 5-HT_{2A} receptor rather than the 5-HT_{2C} receptor. Pretreatment of the slices with MDL 11,939 (1.5 μM) prevented the effects of α-Methyl-5-HT. Thus, the frequency of sIPSCs before and after the application is 2.23 ± 0.31 and 2.61 ± 0.55, respectively (n=8, p>0.05, Figure 3A, B, E). Similarly, the pretreatment of the slices with MDL 100, 907 (150 nM) also prevented the effects of α-Methyl-5-HT. The frequency of sIPSCs before and after the application is 2.56 ± 0.30

and 2.67 ± 0.23, respectively (n=16, p>0.05, Figure 3C, D, E). In order to exclude the involvement of 5-HT_{2B} and 5-HT_{2C} receptors in the effects of α-Methyl-5-HT, the 5-$HT_{2B/2C}$ receptor antagonist, SB 206553 (5 μM), was incubated with slices for 30 minutes. The presence of this antagonist did not significantly diminish the effects of α-Methyl-5-HT. Thus, in the presence of SB 206553, the application of α-Methyl-5-HT increased the frequency of sIPSCs to 598. 2 ± 73% of the baseline values (n=6, p>0.05 compared to α-Methyl-5-HT alone, Figure 3E).

Also, the selective 5-HT_{2B} receptor agonist and 5-HT_{2C} receptor agonist had no facilitatory effect on sIPSCs. The frequency of sIPSCs after application of the selective 5-HT_{2B} receptor agonist, BW 723C86 (15 μM), and the selective 5-HT_{2C} receptor agonist, WAY 629 (30 μM), was 94 ± 3.5 % (n=10) and 100 ± 2.5% (n=13) of the baseline values, respectively (Figure 4). This evidence further supports the contention that the 5-HT_{2A} receptor is responsible for the serotonergic facilitation of GABAergic synaptic transmission in the BLA.

3. 5-HT_{2A} receptors were mainly localized to the soma and dendrites of interneurons in the BLA.

Activation of 5-HT_{2A} receptors could enhance GABAergic synaptic transmission through a presynaptic or a postsynaptic mechanism. To distinguish between these two possibilities, we examined the effects of α-Methyl-5-HT in the presence of TTX (1 μM), which blocks action potential-dependent release of GABA. Under this condition, release events should be composed exclusively of single vesicle release events (miniature IPSCs, mIPSCs). As illustrated in Figure 5, in the presence of TTX, the administration of α-

Methyl-5-HT had no effect on the frequency of mIPSCs (n=8, p>0.05). Similarly, α-Methyl-5-HT also failed to change the amplitude of mIPSCs (Figure 5C). These results indicate that α-Methyl-5-HT act on GABAergic interneurons (presynaptic) rather than recorded pyramidal neurons (postsynaptic) to facilitate GABA release.

The enhancement of sIPSCs by serotonin could be due to a depolarizing effect via the activation of somatodendritic 5-HT_{2A} receptors on GABAergic neurons and/or due to a direct effect at GABAergic terminals. The fact that α-Methyl-5-HT had no effect on mISPCs tends to support the mechanism that 5-HT acts on the somatodendritic 5-HT_{2A} receptors on GABAergic neurons to depolarize and excite them. Indeed, as illustrated in Figure 6, immunofluorescence signals for 5-HT_{2A} receptors are primarily localized to the cell body and dendrites. 5-HT_{2A} signal-positive cell bodies appear to be interneuron-like and the majority of signals for the 5-HT_{2A} receptor (89%) overlap with the signals for the interneuron marker parvalbumin (see Figure 6).

5-HT_{2A} receptors are normally coupled to Gq/11 protein, which then is further coupled to phospholipase C (PLC). In order to determine whether or not 5-HT_{2A} receptor-mediated effects in the BLA interneurons depends on PLC activation, we incubated the slices with a PLC inhibitor, U73122 (20 μM), for at least 30 minutes. In the presence of this inhibitor, application of α-Methyl-5-HT (15 μM) enhanced the frequency and amplitude of sIPSCs to 608.4 ± 41.0% and 169.7 ± 19.4% of the baseline value, respectively (n=9, Figure 7), which is not significantly different from the effect of α-Methyl-5-HT alone. The data suggest that the effect is independent of PLC activation.

4. Stress impaired serotonergic facilitation of GABAergic synaptic transmission in BLA.

To determine whether 5-HT_{2A} receptor-mediated serotonergic facilitatory effects in the BLA is vulnerable to stress, we randomly assigned rats into control and stress groups. After three days, the rats were sacrificed for electrophysiological studies. As illustrated in Figure 8A, the parameters of sIPSCs including the frequency, amplitude, rise time and decay time constant were not significantly changed by exposure to three-day stress compared to control animals. The frequency of sIPSCs in control and stress is 3.03 ± 0.65 HZ and 3.15 ± 0.89 HZ, respectively. The amplitude is 33.16 ± 2.10 pA and 31.70 ± 1.44 pA, respectively. However, the facilitatory effects of α-Methyl-5-HT on sIPSCs were impaired by stress. As illustrated by Figure 8 C, D, E, application of α-Methyl-5-HT (15 μM) enhanced the frequency of sIPSCs only to 260.7 ± 25.70% of the baseline values in stressed animals, which is significantly different from the effect of α-Methyl-5-HT at the same concentration in the control animals (601.0 ± 39.4% of the baseline values). Such a difference became more evident at the higher concentration. At 100 μM, α-Methyl-5-HT increased the frequency by only 440.5 ± 48.0% in stressed amygdala slices, which appears to reach its maximum effect. In control slices, the same concentration of α-Methyl-5-HT enhanced the frequency by 1160.5 ± 99.1%, which still did not reach its maximum effect. Dose-response curve for control and stressed animals shows that the maximum effect of α-Methyl-5-HT has been decreased by exposure to stress, indicating that BLA 5-HT_{2A} receptor signaling has been impaired by exposure of rats to stress (Figure 8E).

In order to further confirm such stress-induced impairment of 5-HT_{2A} receptor signaling in the BLA, the mRNA and protein levels of the 5-HT_{2A} receptor after stress were examined. As illustrated in Figure 9A, exposure to three-day stress caused a significant decrease in mRNA level of 5-HT_{2A} receptors in the BLA and hippocampus compared with control rats, while the mRNA level of 5-HT_{2C} receptors was not significantly changed by exposure to stress in the both BLA and hippocampus. Representative western blots of 5-HT_{2A} receptors in rat BLA are shown in Figure 9B. The molecular mass of 5-HT_{2A} receptors was 55 kDa. As illustrated by Figure 9B, the protein level of 5-HT_{2A} receptors was significantly decreased in the BLA but not in the hippocampus of stressed rats as compared with control rats. These data indicated that stress downregulated 5-HT_{2A} receptors in the BLA, compatible with the electrophysiological data.

5. 5-HT_{2A} receptor blockade before or immediately after stress prevents stress-enhanced ASR.

A characteristic behavioral change resulting from the stress protocol used in this study is enhanced ASR, and this behavioral change is associated with decreased GABAergic transmission in the amygdala (Hitchcock et al., 1989;Sananes and Davis, 1992;Adamec et al., 1999;Gewirtz et al., 1998;Davis et al., 1997b;Davis et al., 1997c;Van Nobelen and Kokkinidis, 2006). In order to examine whether deficit in BLA GABAergic transmission resulting from 5-HT_{2A} receptor downregulation is involved in this enhanced ASR, we blocked the 5-HT_{2A} receptor with MDL 11,939 before or immediately stress, which is assumed to prevent BLA 5-HT_{2A} receptors from being downregulated. Since

previous studies indicated that enhanced ASR became evident on Day 10 after stress (Servatius et al., 1995;Ottenweller et al., 1989;Khan and Liberzon, 2004), we measured ASR on Day 10 and Day 21 after stress.

Consistent with previous reports, stress increased the ASR amplitude at Day 10 after stress (data not shown), and vehicle injection did not affect the stress-enhanced effect on ASR [$f(1, 14) = 4.84$, $p \leq 0.05$] (Figure 10A, B). The effect of stress on ASR still remained at Day 21 after stress [$f(1, 14) = 10.84$, $p \leq 0.01$]. Although the 5-HT_{2A} antagonist is without effect on ASR amplitude in control animals (Vollenweider et al., 2007;Barr et al., 2004;Varty et al., 1999), MDL 11,939 injected prior to stress dose-dependently decreased stress-enhanced startle response to both stimuli (100 dB and 110 dB) on both Day 10 [$F(2, 43) = 5.763$, $p \leq 0.01$] and Day 21 [$F(2, 44) = 4.103$, $p \leq 0.05$] after stress, as illustrated in figure 10A, 10B. At the dose of 1.5mg/kg of MDL 11,939, ASR was reduced to normal compared to the control animals on both Day 10 and Day 21 after stress. MDL 11,939 at 1.5mg/kg injected immediately after stress also decreased the stress-induced enhanced ASR to both stimuli ($n = 8$, $p \leq 0.01$ on Day 10 and $p \leq 0.05$ on Day 21 for both stimuli).

DISCUSSION

This study, for the first time, demonstrated that the 5-HT_{2A} receptor is responsible for the serotonergic facilitation of GABAergic synaptic transmission in the BLA. Our data also, for the first time, showed that this 5-HT_{2A} receptor-mediated serotonergic facilitatory effect on GABA release can be impaired by stress. In addition, receptor blockade during stress could prevent stress-enhanced ASR, suggesting that alteration in the BLA 5-HT_{2A} receptor signaling may be responsible for the occurrence of enhanced ASR in stressed subjects.

5-HT_{2A} receptors mediate the serotonergic facilitation of GABA release in BLA

Although previous studies have shown that the primary action of serotonin in the BLA is to facilitate GABA release to increase the inhibitory tone over the projection neurons (Rainnie, 1999;Stutzmann et al., 1998;Stutzmann and LeDoux, 1999), the subtypes of the serotonin receptor involved in this serotonergic facilitatory effect remain inconclusive. It has been implicated that the 5-HT_2 receptor is involved in the serotonergic facilitatory effect (Rainnie, 1999;Stein et al., 2000). Previous studies also showed that the activation of the presynaptic 5-HT_3 receptor in dissociated neurons of the BLA facilitates GABA release (Koyama et al., 2002;Koyama et al., 2000). However, using amygdala slice preparation, our results showed that the 5-HT_3 receptor agonist 2-Methyl-5-hydroxytryptamine (15μM) had virtually no facilitatory effect on GABA

release, suggesting that the 5-HT_3 receptor is unlikely to be a key contributor of serotonergic facilitatory effect on GABA release in the BLA (data not shown). Indeed, the 5-HT_3 receptor was shown to be localized only to subsets of GABAergic terminals of BLA interneurons (Koyama et al., 2002), and this receptor could be quickly desensitized by the bath perfusion of agonists (Belelli et al., 1995), which may explain the lack of effect of 2-Methyl-5-hydroxytryptamine on GABA release in our study.

Our results indicate that the primary serotonin receptor involved in the serotonergic facilitation of GABA release is the 5-HT_{2A} receptor. First of all, the broad 5-HT_2 receptor agonist α-Methyl-5-HT strongly facilitated the GABAergic synaptic transmission in almost every pyramidal cell recorded (105/123, 85%). Since α-Methyl-5-HT is also a good agonist for the 5-HT_4 receptor (Xiang et al., 2005;Bockaert et al., 1992;Gerald et al., 1995) and may have some affinity for the 5-HT_7 receptor (Amireault and Dube, 2005), involvement of these two receptors is possible. However, the 5-$HT_{2A/2C}$ receptor antagonist Ketanserin completely blocked the effect of α-Methyl-5-HT, excluding the involvement of other serotonin receptors. There are three subtypes of the 5-HT_2 receptor, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} (Saudou and Hen, 1994). Our results support the involvement of the 5-HT_{2A} receptor but not the 5-HT_{2B} and 5-HT_{2C} receptor. First, MDL 11,939, completely blocked the effects of α-Methyl-5-HT (15μM) at a concentration as low as 1.5μM. MDL 11,939 has about a 160-fold higher affinity for the 5-HT_{2A} receptor than the 5-HT_{2C} receptor (K_i = 0.54 nM for the 5-HT_{2A} receptor versus Ki = 81.6 nM for the 5-HT_{2C} receptor)(Dudley MW, 1988) and has been used to distinguish the 5-HT_{2A} receptor from the 5-HT_{2C} receptor (Harvey et al., 2004;Aloyo et al., 2001). Secondly, MDL 100,907, a more selective 5-HT_{2A} receptor antagonist with

300 times higher affinity for the 5-HT_{2A} receptor than the 5-HT_{2C} receptor (Kehne et al., 1996), also completely blocked the effects of α-Methyl-5-HT (15μM) at the concentration as low as 150 nM. Thirdly, the 5-$HT_{2B/2C}$ receptor antagonist, SB206553, could not significantly diminish the facilitation of GABA release by α-Methyl-5-HT. Furthermore, the selective 5-HT_{2B} receptor agonist, BW 723C86, and the selective 5-HT_{2C} receptor agonist, WAY 629, had no effect on GABA release in the BLA, further excluding the involvement of 5-HT_{2B} and the 5-HT_{2C} receptors. Taken together, the 5-HT_{2A} receptor is the primary receptor subtypes involved in serotonergic facilitation of GABA release in the BLA.

Since a previous study has shown that serotonin could depolarize the interneurons in the BLA (Rainnie, 1999), the 5-HT_{2A} receptor is very likely somatodendritically localized, and activation of the 5-HT_{2A} receptor at this location by serotonin would depolarize and excite GABAergic interneurons. Indeed, our data support this contention because the majority of 5-HT_{2A} receptor immunofluorescence was localized to soma and dendrites. Alternatively, since receptor activation could also facilitate sIPSCs by enhancing the probability of GABA release from axon terminals, it is possible that 5-HT_{2A} receptors could act cooperatively at the somatodendritic level and at axonal terminals to enhance GABAergic synaptic transmission. However, because α-Methyl-5-HT is essentially without effect on mIPSCs frequency, α-Methyl-5-HT should not act on axons terminals to facilitate GABA release.

The 5-HT_{2A} receptor is one of the Gq/11 protein-coupled receptors (Saudou and Hen, 1994). In general, Gq coupled receptors enhance neuronal excitability by inhibition of membrane leak potassium channels (TASK channels) through its downstream

signaling (Goldstein et al., 2001;Talley et al., 2003). In addition, increasing evidence has shown that inhibition of the TASK channels appears to be independent of activation of PLC, the downstream of Gq/11 protein (Chen et al., 2006;Boyd et al., 2000). The 5-HT_{2A} receptor mediated effect on GABAergic interneurons in the BLA in the present study was independent of PLC activation since blockade of PLC with U73132 did not affect facilitation of GABA release by α-Methyl-5-HT. There is evidence showing that dissociated Gq/11 alpha subunit resulting from activation of such Gq/11 protein-coupled receptors as the 5-HT_{2A} receptor, could directly inhibit TASK channels (Chen et al., 2006).

5-HT_{2A} receptor-mediated serotonergic facilitation of GABA release could be severely impaired by stress.

Excessive or repeated stress can produce long-lasting functional alterations in the amygdala circuitry. The long lasting changes in the efficacy of synaptic transmission in the amygdala have been observed after different types of stress (Adamec et al., 2001;Davis et al., 1994;McKernan and Shinnick-Gallagher, 1997;Kavushansky et al., 2006;Vouimba et al., 2006). In human patients with stress-related affective disorders, the amygdala exhibits increased levels of basal activity (Drevets, 1999), or exaggerated responses to fearful stimuli (Rauch et al., 2000a). The GABAergic system in the BLA is also subject to change after stress, and stress always tends to attenuate the GABAergic systems in the BLA (Braga et al., 2004;Rainnie et al., 2004;Rodriguez Manzanares et al., 2005;Shekhar et al., 2005). The present study reveals another important mechanism by which stress attenuates the inhibitory control over neuronal excitability in the BLA.

Repeated restraint/tail-shock stress produced a severe impairment in 5-HT_{2A} receptor-mediated facilitation of GABA release in the BLA. Given that the baseline of GABAergic activity in the stressed amygdala had no significant difference from that in control animals, stress-induced attenuation of 5-HT_{2A} receptor-mediated GABA release should result from impaired 5-HT_{2A} receptor signaling other than the GABAergic system *per se*. Our qRT-PCR data and western blot data showed that stress not only decreased the mRNA level of the 5-HT_{2A} receptor but also the level of expressed receptors, indicating that the 5-HT_{2A} receptor is downregulated by stress.

Such impaired 5-HT_{2A} receptor signaling after inescapable stress should result from receptor adaptation after prolonged agonist stimulation. Inescapable shock stress could dramatically enhance serotonin level in the amygdala and this enhancement could last at least 2 days after the end of stress (Amat et al., 1998;Maier and Watkins, 2005;Minor and Hunter, 2002). Prolonged stimulation of the 5-HT_{2A} receptor by excess serotonin may result in adaptation of this receptor (i.e., receptor desensitization, internalization and downregulation). Indeed, receptor desensitization and downregulation after chronic agonist stimulation is a common phenomena for 5-HT_{2A} receptor, and has been observed in both culture (Roth et al., 1995) and *in vivo* systems (Damjanoska et al., 2004;Anji et al., 2000;Smith et al., 1999). Interestingly, the 5-HT_{2C} receptor, although coupled to the similar signal pathway as the 5-HT_{2A} receptor, appears to be resistant to chronic agonist stimulation (Smith et al., 1999). This may explain the observation that stress only decreased the mRNA level of the 5-HT_{2A} receptor without affecting the 5-HT_{2C} receptor in the BLA.

5-HT_{2A} receptor blockade prevents stress-induced enhanced ASR.

Our studies, from both functional and molecular perspectives, indicated that the 5-HT_{2A} receptor mediated serotonergic inhibition over the amygdala's excitability could be impaired by stress. Indeed, downregulation of the 5-HT_{2A} receptor in the amygdala after inescapable shock has also been implicated in previous studies, and it is specific to this stress protocol (restraint or shock alone have no effect) (Petty et al., 1997;Wu et al., 1999). These studies also implicated that downregulation of this receptor appears to be long-lasting (Dwivedi et al., 2005;Petty et al., 1997). Our preliminary qPCR data also showed that 10 days after stress, the mRNA level of the 5-HT_{2A} receptor in the amygdala was still lower than control (data not shown). Considering that the protein turnover is much slower than the mRNA turnover, these observations indicate that impaired BLA 5-HT_{2A} receptor signaling still exist 10 day after stress and may last longer. Thus, such impairment of 5-HT_{2A} receptor-mediated serotonergic inhibition may contribute to behavioral, emotional sequelae resulting from stress, including enhanced ASR (Servatius et al., 1995). To examine this association, the 5-HT_{2A} receptor antagonist MDL 11,939 was administrated before or immediately after stress. Since the BLA 5-HT_{2A} receptor downregulation should result from prolonged stimulation by stress-elevated 5-HT, and antagonists can prevent agonist-induced receptor downregulation (Brown et al., 1998;Bieck et al., 1992;Baumhaker et al., 1993;Horackova et al., 1990;Lassegue et al., 1995;Scarceriaux et al., 1996;Chau et al., 1994;Fukamauchi et al., 1993), MDL 11,939 treatment would prevent BLA 5-HT_{2A} receptors from being downregulated by stress. If certain stress-induced changes such as enhanced ASR do result from impaired BLA 5-HT_{2A} receptor signaling, the administration of MDL 11,939 should be able to prevent

such stress-enhanced ASR. The results do support such a hypothesis. Our data showed that the low dose of MDL 11, 939, but not vehicle, reduced startle amplitude toward control levels. At a higher dose, MDL 11, 939 completely reversed stress-enhanced startle, whether it was administrated before or immediately after stress.

If impaired 5-HT_{2A} receptor signaling in the BLA does contribute to the occurrence of enhanced ASR, why does stress-induced enhanced ASR appear only after 10 days (Servatius et al., 1995)? Although the reasons of delayed enhanced ASR after inescapable shock have not been well documented in the literature, certain studies implicate that this delay is due to muscle fatigue and locomotor impairment after restrained stress (Ottenweller et al., 1989;Ottenweller et al., 1994;Ottenweller et al., 1992;Servatius et al., 1995). Thus, the length of the delay was positively related to both the number and intensity of footshocks (Servatius et al., 1995).Therefore, although the pathophysiological mechanisms may have been available to evoke enhanced ASR immediately after stress, the modification of the startle circuitry may not be fully completed until 10 days after stress.

Impaired BLA 5-HT_{2A} receptor signaling may participate in the occurrence of stress-enhanced ASR.

The data presented strongly suggest that malfunctioning of 5-HT_{2A} receptor-mediated facilitation of GABA release in the amygdala may contribute to the occurrence of stress-induced enhanced ASR. It is well accepted that the amygdala circuitry is responsible for the sensitization of ASR and fear-enhanced startle (Koch and Schnitzler, 1997). Enhanced ASR by repeated foot shock and predator exposure is believed to be

mediated by the amygdala (Hitchcock et al., 1989;Sananes and Davis, 1992;Adamec et al., 1999;Gewirtz et al., 1998;Davis et al., 1997b;Davis et al., 1997c;Van Nobelen and Kokkinidis, 2006). Furthermore, this enhanced ASR may be associated with decreased GABAergic transmission in the BLA (Van Nobelen and Kokkinidis, 2006;Stork et al., 2002). Our data support the notion that there is a causal relationship between enhanced ASR and malfunctioning of serotonergic modulation of GABA release in the amygdala. In the normal amygdala, basal levels of serotonin, acting via 5-HT_{2A} receptors, may contribute to tonic inhibition of BLA pyramidal neurons (Stutzmann et al., 1998;Stutzmann and LeDoux, 1999), which allows glutamatergic sensory input more effectively filtered to permit relevant stimuli to be fully processed by the amygdala. Decreased serotonergic functioning might result in deficient GABAergic modulation of excitatory sensory afferents. Defective GABAergic influence may allow innocuous sensory signals to be processed in the amygdala and activate downstream responses. One of the consequences of such an activation is the enhanced startle response (Koch and Schnitzler, 1997). Administration of MDL 11,939 during stress may protect the 5-HT_{2A} receptor from being impaired, and thus maintain an intact serotonergic functioning in the amygdala. As a result, the abnormal startle response could be prevented.

Since MDL 11,939 in this study was administrated systematically, it is possible that MDL 11, 939 may act on other brain sites to prevent the stress-enhanced startle. Previous studies has shown that inescapable stress could also induce 5-HT_{2A} receptor change in the hippocampus (Dwivedi et al., 2005), hypothalamus (Dwivedi et al., 2005;Wu et al., 1999), and prefrontal cortex (Dwivedi et al., 2005;Harvey et al., 2003), suggesting the change of 5-HT_{2A} receptors in these brain sites after stress may also

contribute to the development of severe outcomes of stress. However, the change of 5-HT_{2A} receptors in these brain regions may be associated with other stress-induced abnormalities, but not enhanced ASR. For instance, stress-induced 5-HT_{2A} receptor alterations in the hypothalamus, prefrontal cortex and hippocampus were specifically associated with stress-induced behavioral depression (Dwivedi et al., 2005), while a stress-induced decrease of amygdala 5-HT_{2A} receptor was not related to this behavioral abnormality (Petty et al., 1997;Wu et al., 1999). Also, 5-HT_{2A} receptor signaling in the cortex has shown to be closely associated with cognitive function of working memory (Williams et al., 2002). Thus, alterations of cortical 5-HT_{2A} receptor signaling after stress may be related to stress-induced cognitive deficit (Oei et al., 2006;Morgan III et al., 2006;Liston et al., 2006;McFarlane et al., 2002;Cherrie et al., 2001). A recent study also demonstrated that the intact cortical 5-HT_{2A} receptor signaling is essential for conflict anxiety behaviors in mice (Weisstaub et al., 2006). Nevertheless, this study did not examine the association between cortical 5-HT_{2A} receptor signaling and ASR. Thus, although it could not be excluded that other brain regions may be involved in the effect of MDL11,939 on enhanced ASR, the amygdala should be a primary brain site MDL 11,939 acted on to prevent stress-induced enhanced ASR.

Alternatively, the selective 5-HT_{2A} receptor antagonist MDL 11, 939 may exert its prophylactic effect on stress-enhanced startle by preventing this receptor from being activated during the stress as opposed to protecting the receptor from being impaired. MDL 11, 939 treatments may block long-term 5-HT_{2A} receptor signaling in the corticolimbic structures during stress, while this long-term signaling in the corticolimbic structures may be critical in development of enhanced ASR. Many studies suggested that

the 5-HT_{2A} receptor in the paraventricular nucleus (PVN) is involved in the release of the CRF during stress (Zhang et al., 2002;Mikkelsen et al., 2004;Hanley and Van de Kar, 2003;Van de Kar et al., 2001;Saphier et al., 1995), while the CRF, by acting on the amygdala and/or bed nucleus of the stria terminals (BNST), is a crucial mediator in developing stress-enhanced ASR (Koob et al., 1993;Lee and Davis, 1997;Risbrough et al., 2003;Risbrough and Stein, 2006;Walker and Davis, 1997). MDL 11,939 treatment during stress may attenuate stress-induced enhancement of CRF, and thereby interfere with the development of the pathophysiology associated with enhanced ASR. Nevertheless, our study showed that MDL 11,939 pretreatment could exacerbate stress-induced body weight loss (data not shown), a physiological change that depends on activation of HPA axis (Harris et al., 2006;Smagin et al., 1999), suggesting that 5-HT_{2A} receptor antagonist may not be able to attenuate stress-induced enhancement of CRF. To examine whether MDL 11, 939 treatments can alter activation of HPA axis during stress may be able to better appreciate the role of the hypothalamic 5-HT_{2A} receptor in the preventive effect of MDL 11,939 on enhanced ASR.

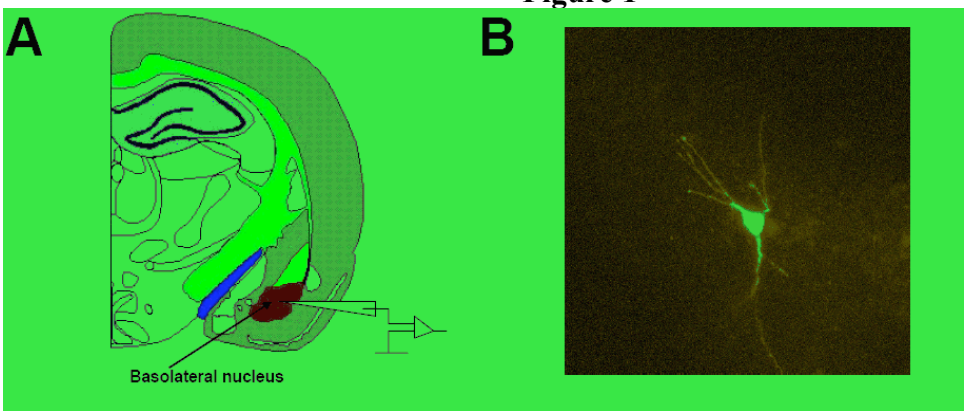
Functional implications.

Stress-associated psychiatric disorders, such as PTSD, are associated with hyperactivity and hyper-responsiveness of the amygdala (Shin et al., 2006). It has been hypothesized that such alterations in the amygdala are due to the loss of proper cortical modulation of the amygdala, and/or due to a lowered threshold of amygdala response to emotionally significant stimuli (Villarreal and King, 2001). The present findings suggest that a reduction in GABAergic transmission due to impaired 5-HT_{2A} receptor signaling

may be one of the key mechanisms responsible for the apparently reduced threshold of amygdala's activation in these psychiatric disorders. Indeed, it appears that there is a significant association between polymorphism of the 5-HT_{2A} receptor and panic disorder (Inada et al., 2003). The change of the 5-HT_{2A} receptor has also been observed in stress-associated psychiatric disorders, such as depression (Sheline et al., 2004;Mintun et al., 2004) and suicide associated with depression (Oquendo et al., 2006;Rosel et al., 2004;van Heeringen et al., 2003;Pandey et al., 2002;Rosel et al., 2004). These findings strongly suggest an important role of the 5-HT_{2A} receptor in the pathogenesis of these stress-related disorders. Also, the present study suggests that the 5-HT_{2A} receptor antagonist MDL 11,939 may be a good prophylactic agent for stress-associated psychiatric disorders, such as PTSD.

Figure 1. Effect of 5-HT on sIPSCs in the basolateral amygdala. **A.** The representive coronal brain slice containing the basolateral nucleus (red), where neurons were recorded**.** **B**. Photomicrograph of a pyramidal cell showing the typical morphology of the recorded neurons. The cell has been labeled with Lucifer Yellow. **C.** In the presence of the 5-HT_3 receptor antagonist, MDL 72222 (20μM), and the 5-HT_1 receptor antagonist, cyanopindolol (20μM), administration of 5-HT (50 μM) dramatically enhances the frequency of sIPSCs and shifts their amplitude distribution toward larger sizes in a BLA neuron. **D**. Cumulative probability plots of inter-event intervals and amplitude of sIPSCs (same cell as in the top trace). **E**. Pooled data (mean ± S.E.M.) from 9 neurons. The bar graph on the left shows the 5-HT-induced changes in amplitude, frequency of sIPSCs. The bar graph on the right panel shows the time course of changes in sIPSCs frequency during the application of 5-HT. (*$p<0.05$, **$p<0.01$)

Figure 1


A
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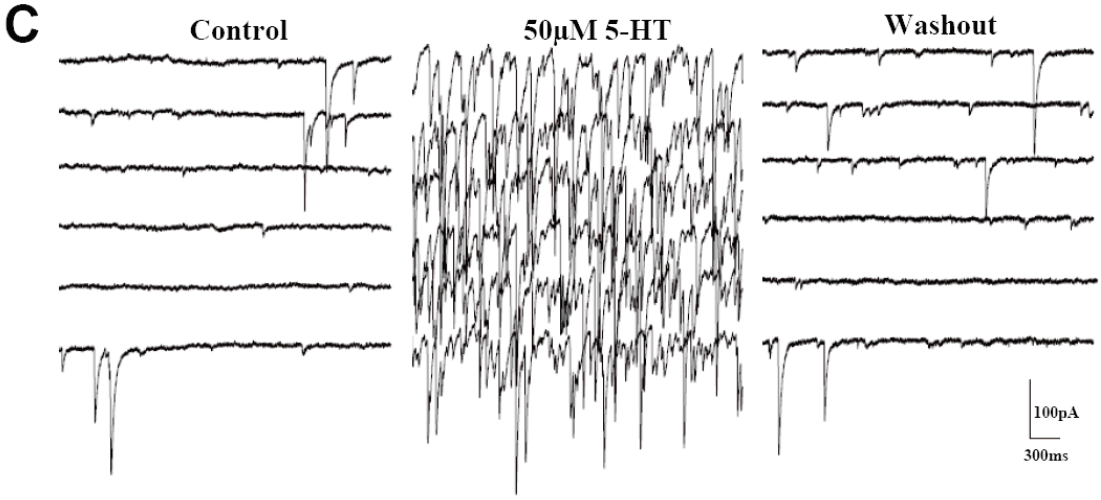
Basolateral nucleus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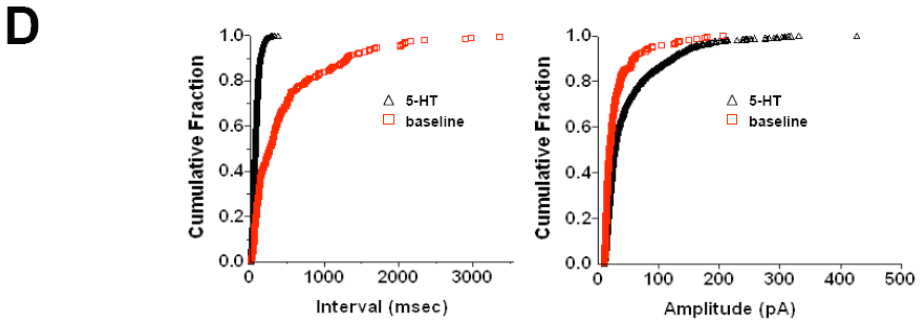
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Amplitude (pA)
5-HT
baseline

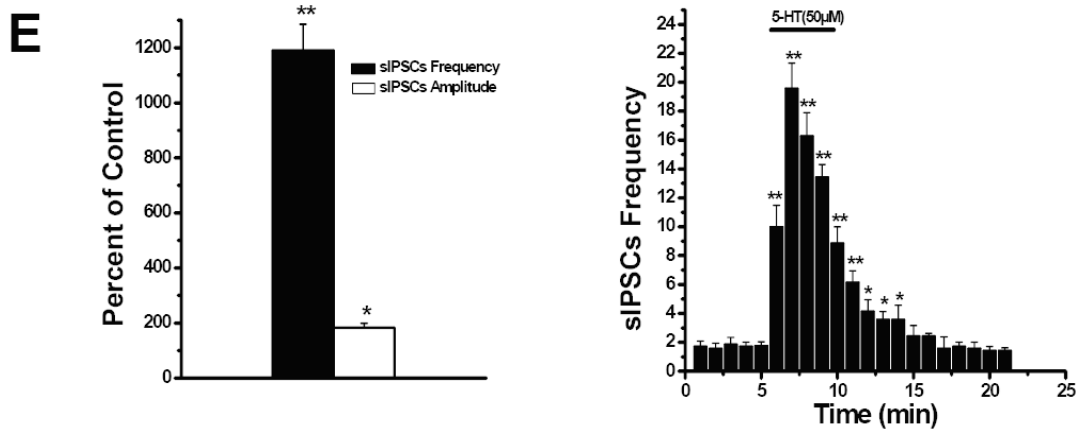

Figure 2

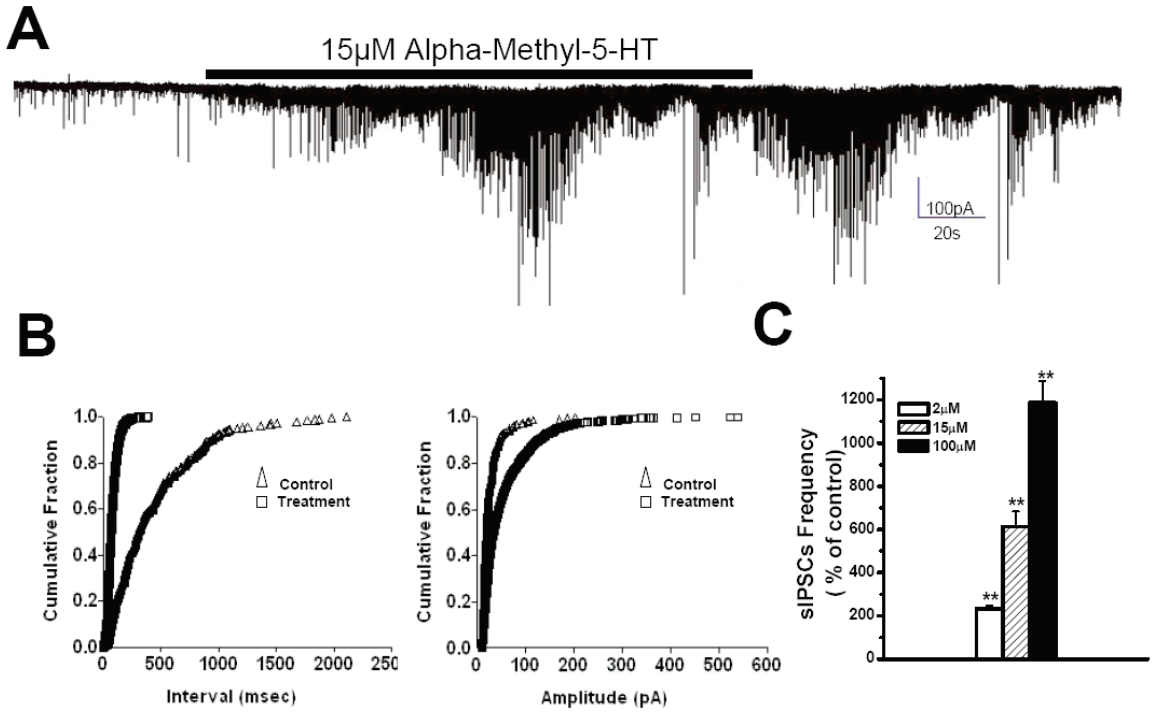

Figure 2. The 5-HT$_2$ receptor agonist α-Methyl-5-HT dose-dependently facilitated the sIPSCs in the BLA. **A** shows a sample neuron where administration of α-Methyl-5-HT (15μM) dramatically enhances the frequency of sIPSCs and shifts their amplitude distribution toward larger sizes. **B**. Cumulative probability plots of inter-event intervals and amplitude of sIPSCs (same cell as in the top trace). **C**. The bar graph show the group

data of the effect of α-Methyl-5-HT on sIPSC frequency (n=10 for each concentration of α-Methyl-5-HT, **p≤0.01).

Figure 3. The influence of different 5-HT$_2$ receptor antagonists on the facilitatory effect of α-Methyl-5-HT on sIPSCs. **A** show an example of neuron where α-Methyl-5-HT could not longer induce the facilitation of sIPSCs in the presence of 1.5 μM MDL 11,939 (holding potential is -70 mV). **B**.Cumulative probability plots of inter-event intervals and amplitude of sIPSCs (same cell as in the top trace). **C** show an example of neuron where α-Methyl-5-HT could not longer induce the facilitation of sIPSCs in the presence of 150 nM MDL 100,907 (holding potential is -70 mV). **D**. Cumulative probability plots of inter-event intervals and amplitude of sIPSCs (same cell as in the top trace). **E**. pooled data (mean ± S.E.M.) indicating the effect of α-Methyl-5-HT alone (n=9), the effect of α-Methyl-5-HT in the presence of 10 μM Ketanserin (n=11), the effect in the presence of 1.5 μM MDL 11,939 (n=8), the effect in the presence of 5 μM SB 206553 (n=6) or in the presence of 1.5 μM MDL 100,907 (n=16). (**P<0.01)

Figure 3

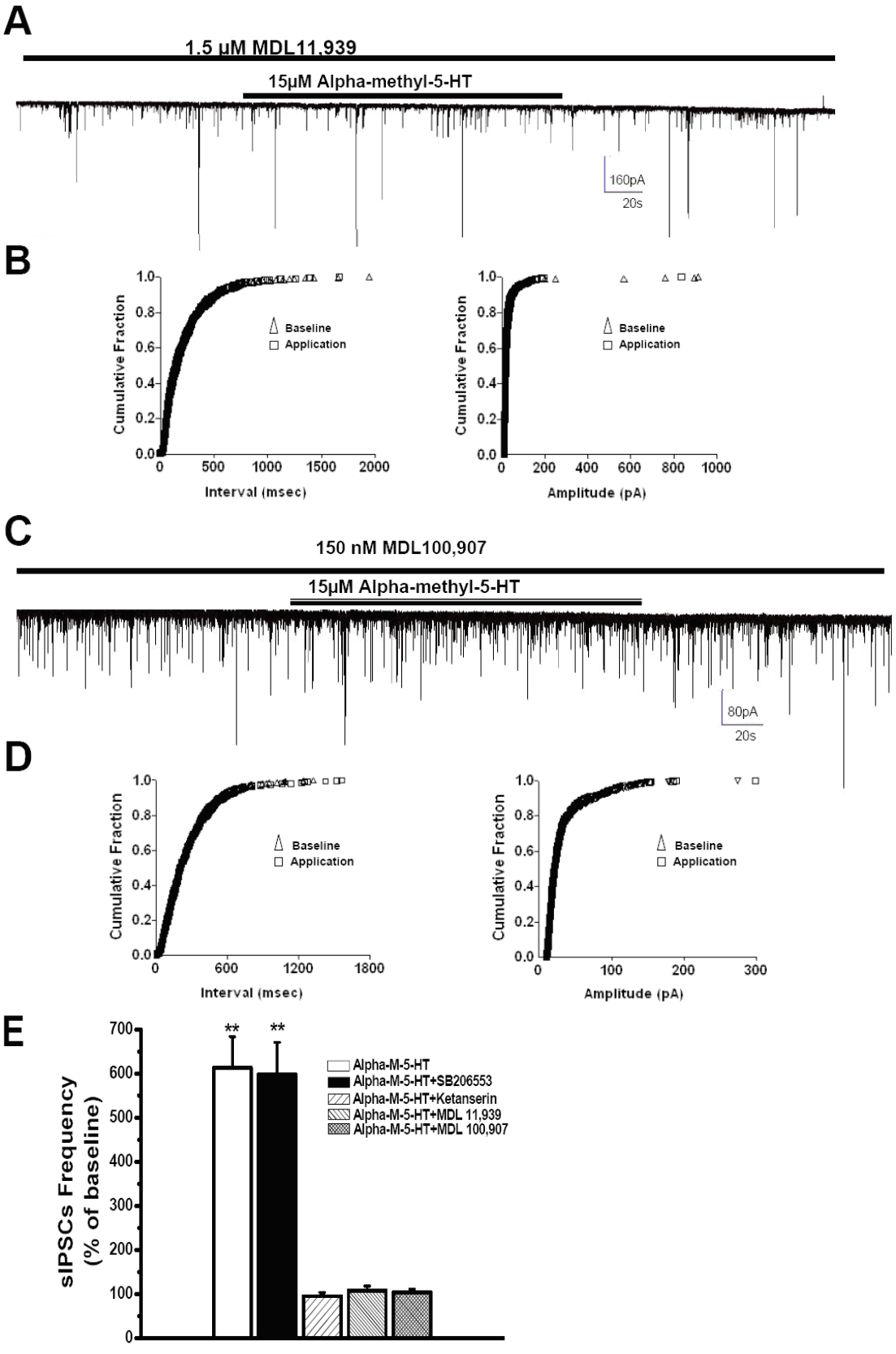
A
1.5 μM MDL11,939
15μM Alpha-methyl-5-HT
160pA
20s
B
Cumulative Fraction
Baseline
Application
Interval (msec)
Amplitude (pA)
C
150 nM MDL100,907
15μM Alpha-methyl-5-HT
80pA
20s
D
E
sIPSCs Frequency (% of baseline)
Alpha-M-5-HT
Alpha-M-5-HT+SB206553
Alpha-M-5-HT+Ketanserin
Alpha-M-5-HT+MDL 11,939
Alpha-M-5-HT+MDL 100,907

Figure 4. The effects of the selective 5-HT$_{2C}$ receptor agonist WAY 629 and the selective 5-HT$_{2B}$ receptor agonist BW 723C86 on sIPSCs in the BLA. **A** shows an example of BLA neurons in which application of the selective 5-HT$_{2C}$ receptor agonist WAY 629 has no effect on sIPSCs. **B**. Cumulative probability plots of inter-event intervals and amplitude of sIPSCs (same cell as in A). **C** shows an example of BLA neurons in which application of the selective 5-HT$_{2B}$ receptor agonist BW 723C86 has no effect on sIPSCs. **D.** Cumulative probability plots of inter-event intervals and amplitude of sIPSCs (same cell as in C). **E.** The bar graph shows the group data of the effect of these two agonists on sIPSCs frequency, compared to the effect of α-Methyl-5-HT (n=10 for each agonists, **$p \leq 0.01$).

Figure 4

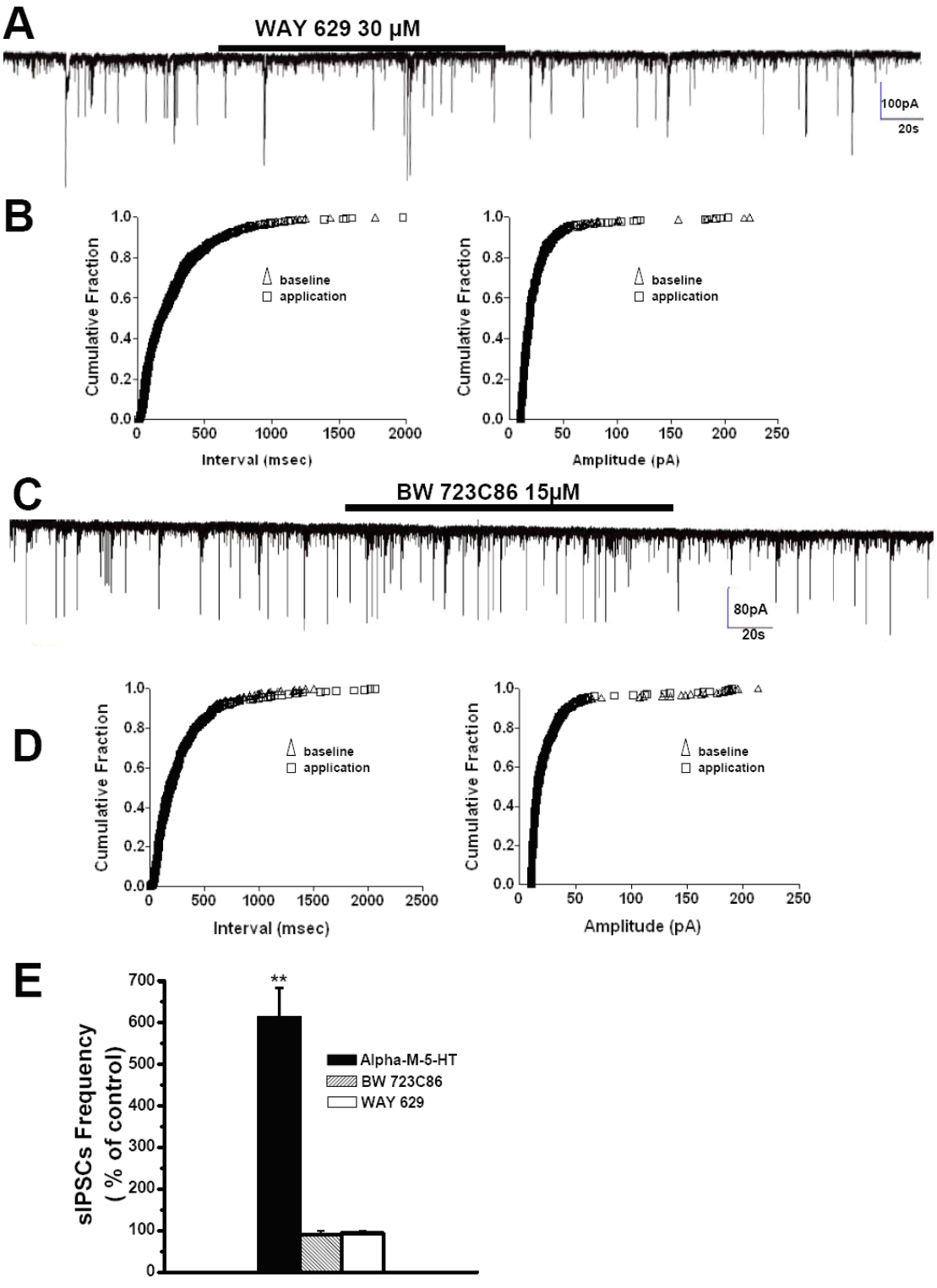
A
WAY 629 30 µM
100pA
20s
B
Cumulative Fraction
baseline
application
Interval (msec)
Amplitude (pA)
C
BW 723C86 15µM
80pA
20s
D
Cumulative Fraction
baseline
application
Interval (msec)
Amplitude (pA)
E
sIPSCs Frequency
(% of control)
**
Alpha-M-5-HT
BW 723C86
WAY 629

Figure 5

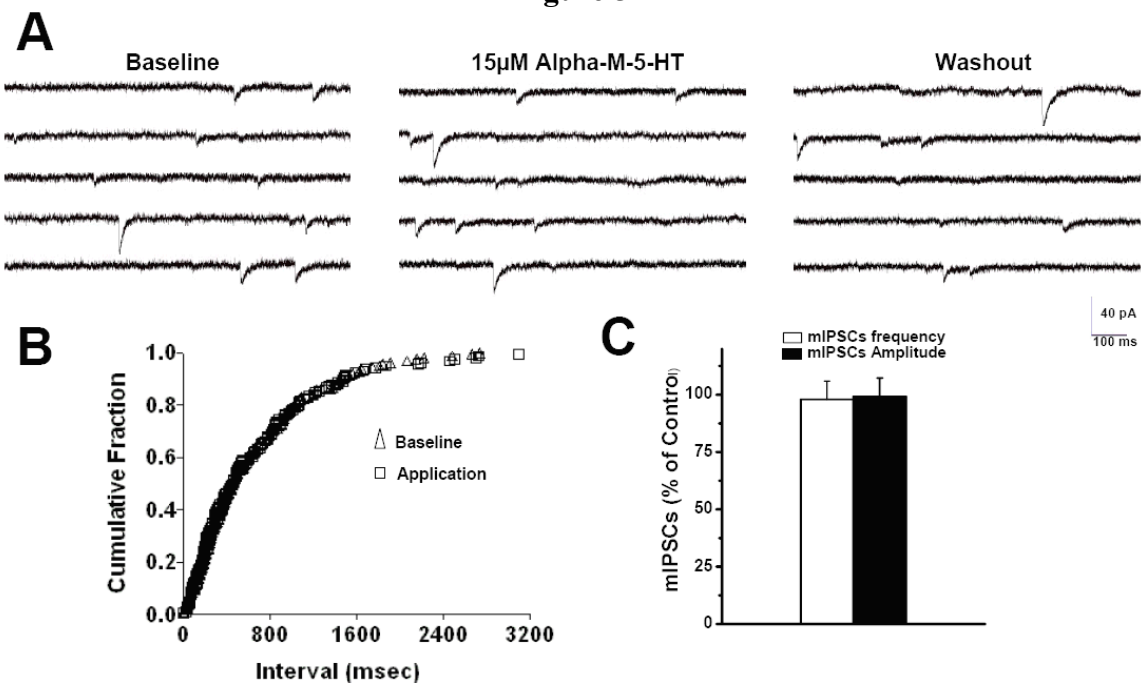

Figure 5. The effect of α-Methyl-5-HT on mIPSCs in the BLA. **A** shows the mIPSCs traces in one cell and administration of α-Methyl-5-HT (15μM) did not induce significantly change of the frequency and amplitude of mIPSCs. **B**. Cumulative probability plots of inter-event intervals of mIPSCs (same cell as in the top trace). C. pooled data (mean ± S.E.M) from 8 neurons. The bar graph shows amplitude and frequency of mIPSCs after application of α-Methyl-5-HT were not significantly different from the baseline.

Figure 6

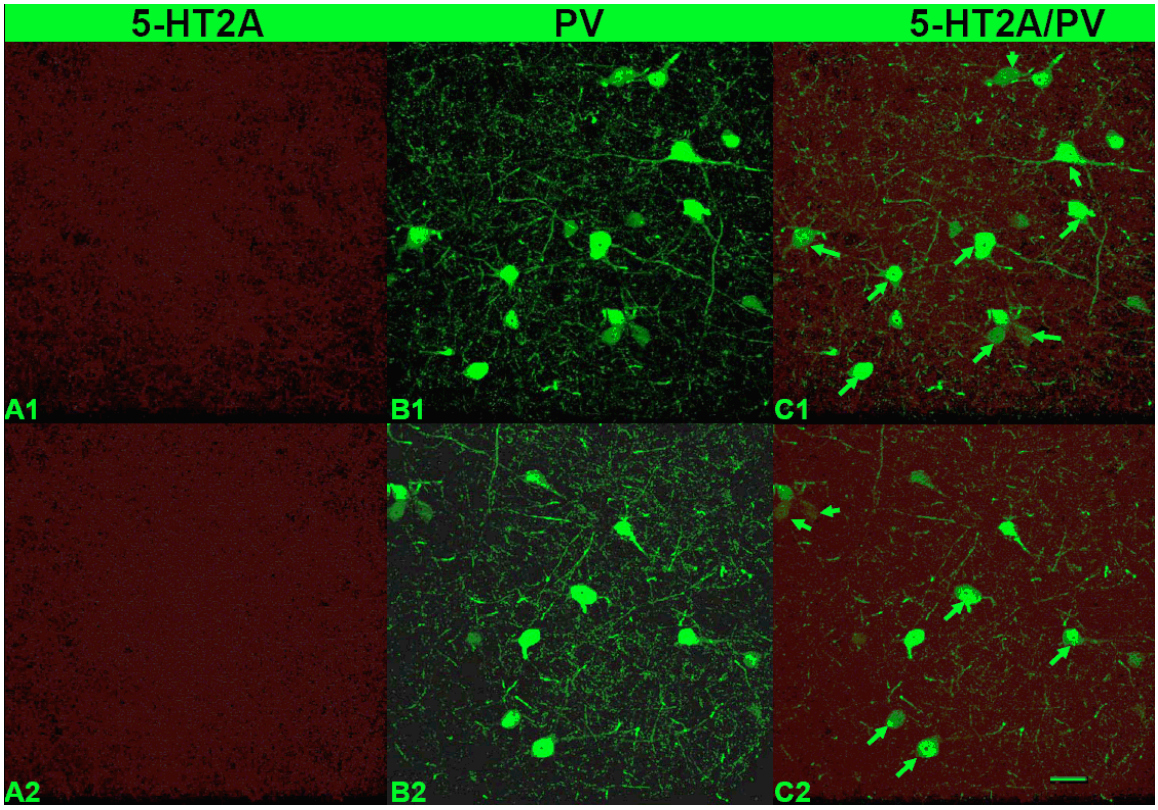

Figure 6. Confocal microscopic images illustrating dual localization of 5-HT$_{2A}$ receptor with parvalbumin (PV) in the BLA. (A1-A2) The signals for the 5-HT$_{2A}$ receptor in the BLA. The majority of signals are localized in the soma and dendrites and the shape of signal-positive cells is interneuron-like. (B1-B2) The signals for interneuron marker PV. (C1–C2) Colocalization of 5-HT$_{2A}$ receptor (red) with PV (green) in the BLA. Arrows in C1-C2 indicates the colocalization of two types of signals (yellow). Scale bar: 25 μm.

Figure 7

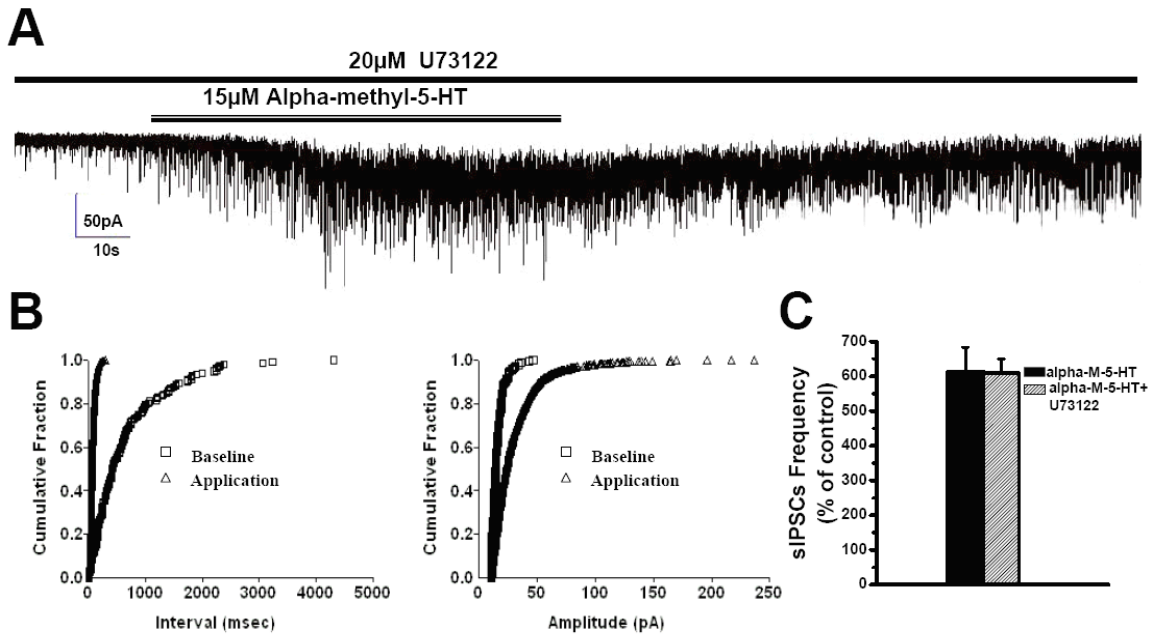

Figure 7.The facilitatory effects of α-Methyl-5-HT on sIPSCs in the presence of PLC inhibitor U73122. **A** show an example of neuron where α-Methyl-5-HT still induced the comparable facilitation of sIPSCs after pretreatment of 20μM U73122 for 30 minutes (holding potential is -70 mV). **B**. Cumulative probability plots of inter-event intervals and amplitude of sIPSCs (same cell as in the top trace). **C**. pooled data (mean ± S.E.M) indicating the effects of α-Methyl-5-HT alone (n=10), and the effects of α-Methyl-5-HT in the presence of 20 μM U73122 (n=9).

Figure 8. Stress attenuated 5-HT$_{2A}$ receptor-mediated facilitatory effect of sIPSCs in the BLA. (**A)**. The data indicate that stress did not significantly affect the frequency, amplitude, rise time and decay time constant of sIPSCs in the BLA (n=23). (**B)**. Up trace: effects of α-Methyl-5-HT (15 μM) on sIPSCs recorded from a BLA pyramidal cell of a control rat. Holding potential: -70mV. Down graphs: cumulative probability plots of interevent intervals and amplitude of sIPSCs, in baseline conditions and during α-Methyl-5-HT application (same cell as in the top trace). (**C**) Up trace: effects of α-Methyl-5-HT (15 μM) on sIPSCs recorded from a BLA pyramidal cell of a stressed rat (holding potential is -70 mV); the effect of α-Methyl-5-HT (15 μM) is significantly smaller than that in control animals. Down graphs: cumulative probability plots of interevent intervals and amplitude of sIPSCs in baseline conditions and during α-Methyl-5-HT application (same cell as in the top trace). (**D).** Pooled data (mean ± S.E.M.) indicating the time course of the effects of α-Methyl-5-HT (15 μM) in both control and animals. Note that stress significantly attenuated the effects of α-Methyl-5-HT (15 μM) at the different time points. (n=18 for stress and n=14 for control, *p≤.05, **p≤0.01). (**E**).The dose response relationship of the effects of α-Methyl-5-HT in control and stress animals. (0.5 μM: n=5 for control and n=4 for stress; 2 μM: n=20 for stress and n=13 for control; 10 μM; n=8 for stress and n=11 for control; 15 μM: n=18 for stress and n=14 for control; 100 μM: n=10 for stress and n=9 for control; 200 μM, n=4 for control and n=5 for stress. *p≤.05, **p≤0.01)

Figure 8

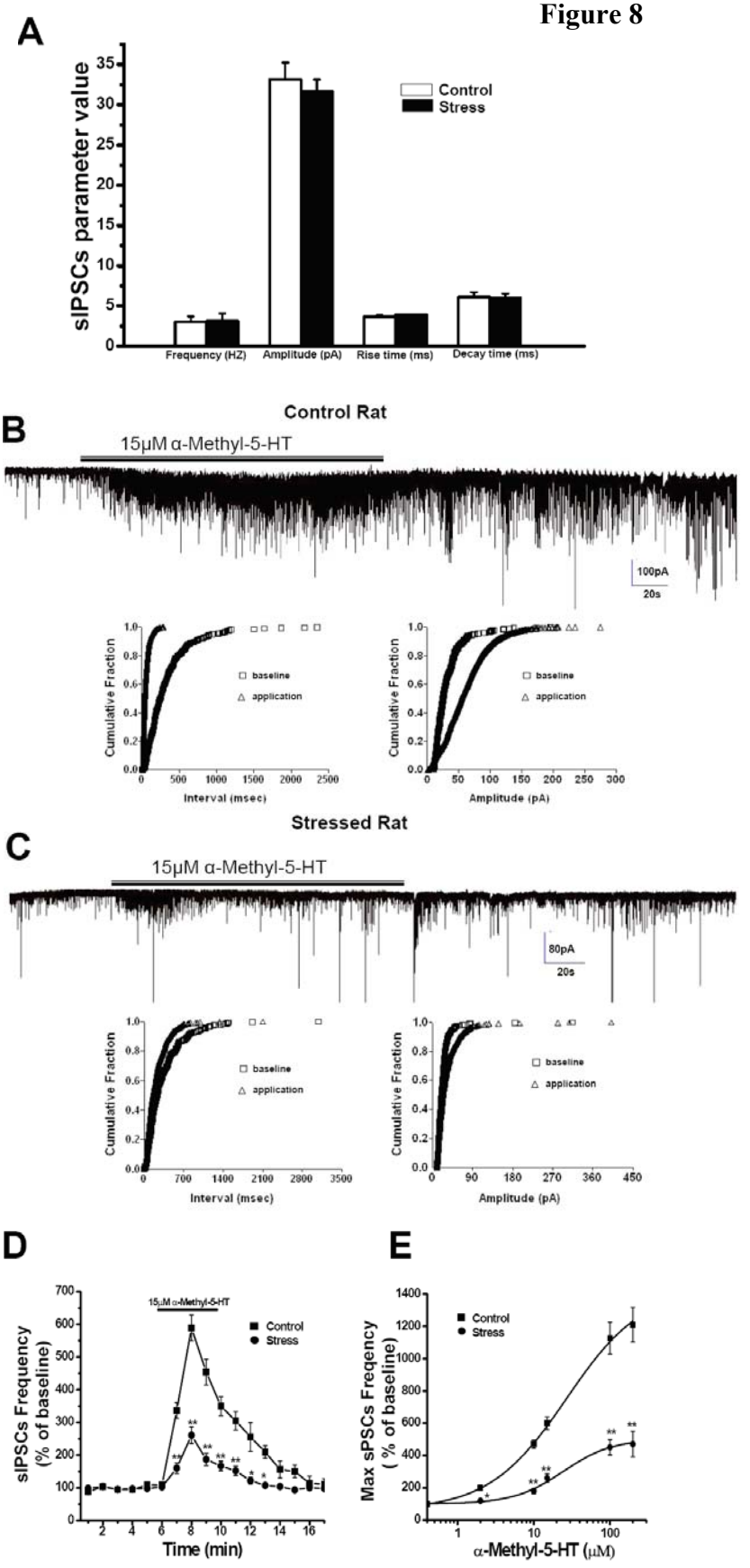
A
sIPSCs parameter value
Control
Stress
Frequency (HZ)
Amplitude (pA)
Rise time (ms)
Decay time (ms)
B
Control Rat
15μM α-Methyl-5-HT
100pA
20s
Cumulative Fraction
baseline
application
Interval (msec)
Amplitude (pA)
C
Stressed Rat
15μM α-Methyl-5-HT
80pA
20s
D
sIPSCs Frequency (% of baseline)
Time (min)
E
Max sPSCs Freqency (% of baseline)
α-Methyl-5-HT (μM)

Figure 9

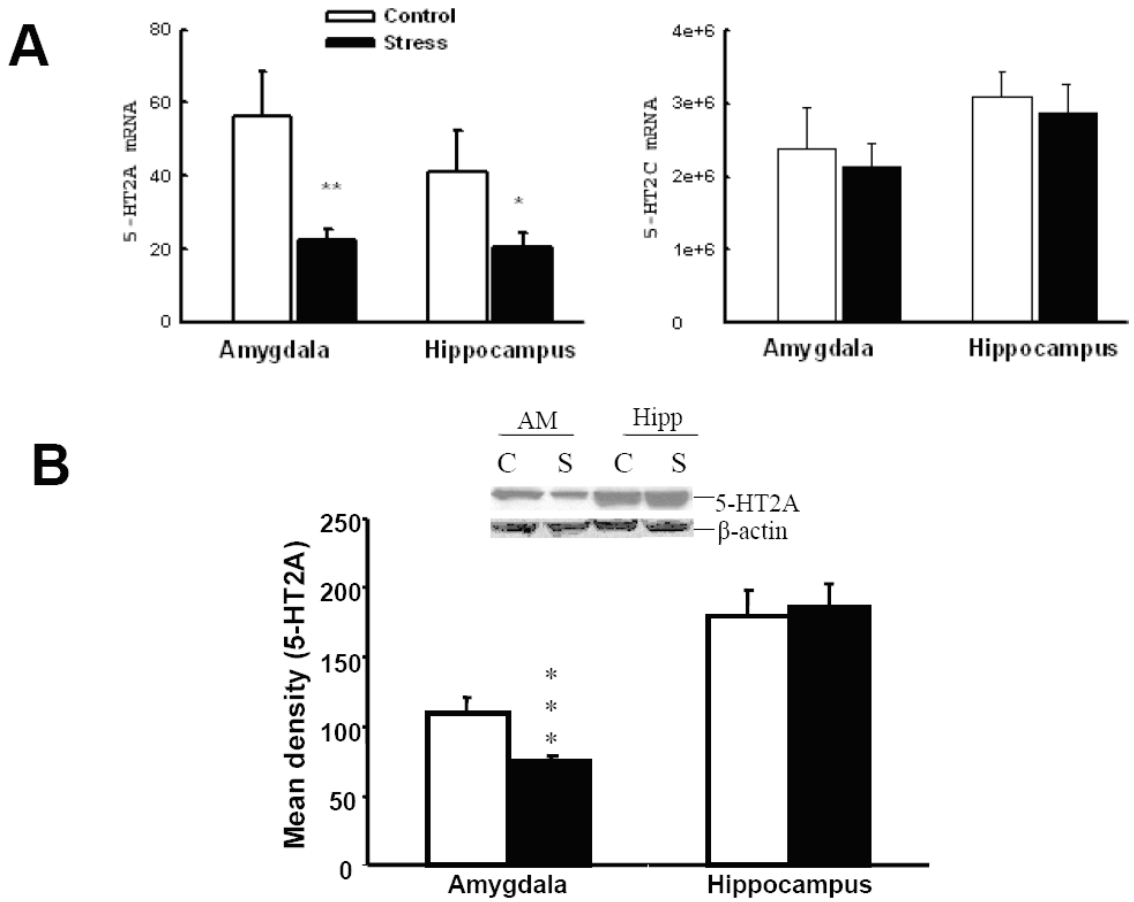

Figure 9. Stress decreased the expression of 5-HT $_{2A}$ receptor mRNA and its proteins in the BLA. (**A**). Stress decreased 5-HT$_{2A}$ receptor mRNA levels in the both BLA and hippocampus, while 5-HT$_{2C}$ receptor mRNA levels in the both BLA and hippocampus were not significantly changed by stress (n=7, *p ≤0.05, **p≤0.01). (**B**). Stress decreased protein levels of BLA 5-HT$_{2A}$ receptors, while 5-HT$_{2A}$ receptors in the hippocampus (Hipp) were not significantly changed by stress. β-actin was used as an internal control. Blot results shown are representative of three separate experiments. ***p<0.001 (stress *vs* control).

Figure 10

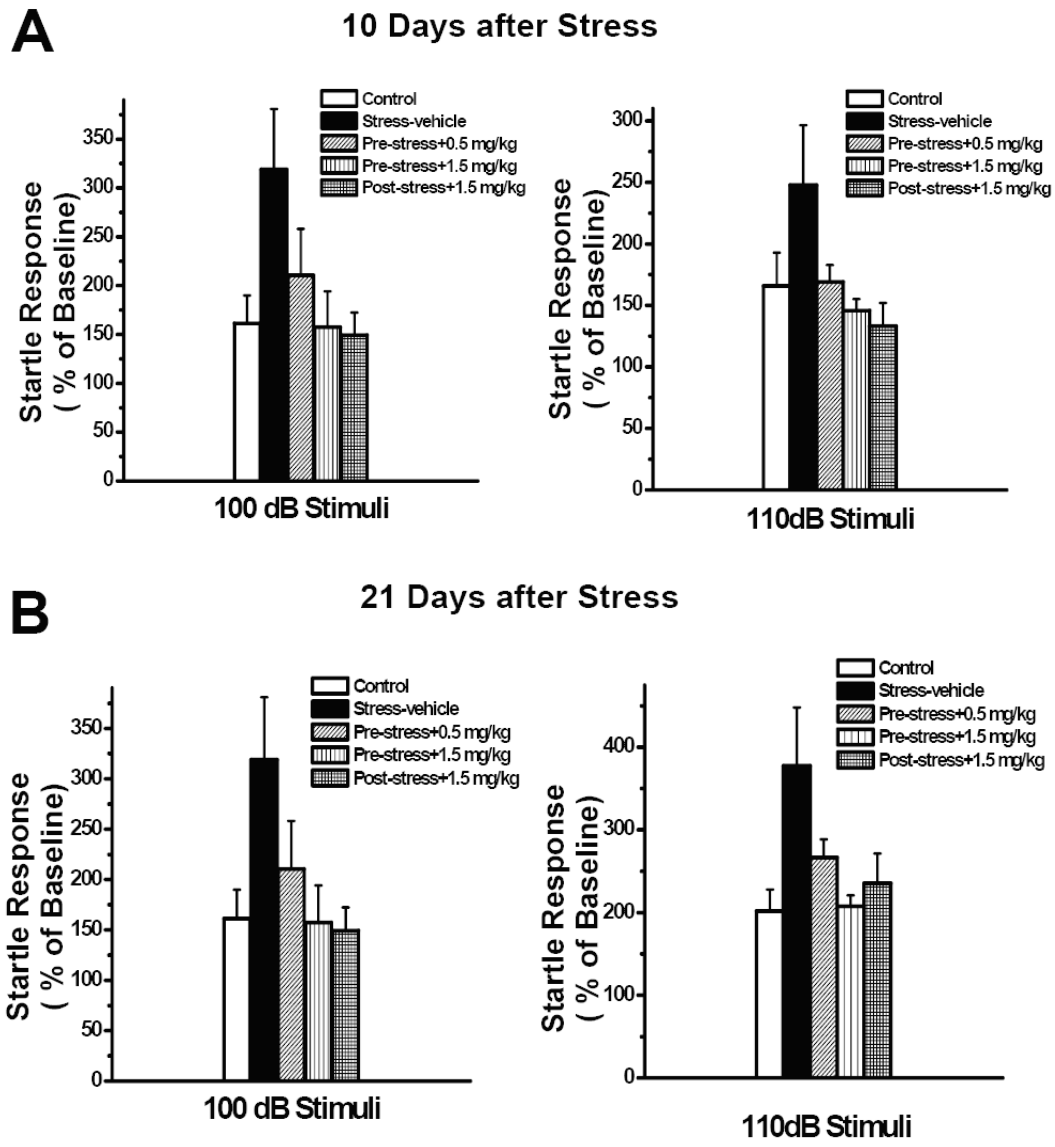

Figure 10. Mean±S.E.M. of peak startle amplitude (represented as % of the baseline of ASR). The groups with MDL 11,939 pretreatment and posttreatment are plotted together. The ASR to 100 dB and 110 dB was tested on both Day 10 after stress (**A**) and Day 21 after stress (**B**).

CHAPTER 3

Stress Downregulates Gene Expression of Hypothalamic 5-HT_{2A} Receptors: Receptor Blockade Reverses Stress-Induced Sustained Body Weight Loss in Experimental Rats

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Keywords: Stress, 5-HT_{2A} receptor, 5-HT_{1B} receptor, MDL 11, 939, startle, body weight, hypothalamus.

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ABSTRACT

Repeated restraint stress can induce long-lasting body weight loss in rodents. Since the serotonergic system in the hypothalamus plays an important role in mediating feeding and body weight, the present study examined if stress-induced sustained body weight loss is associated with alterations of hypothalamic serotonergic system. In rats exposed to three-day restrain-tail shock, the mRNA levels of the hypothalamic 5-HT_{2A} receptors were significantly decreased, while the mRNA levels of 5-HT_{1B} receptors were upregulated by stress. In contrast, the mRNA levels of the hypothalamic 5-HT_{1A} receptors and 5-HT_{2C} receptors remained unchanged. The rats exposed to this stress experienced long-lasting weight loss after cessation of stressful episodes, compared to nonstress control animals. However, injection of the selective 5-HT_{2A} receptor antagonist, MDL 11,939 before stress, although tending to exacerbate initial body weight loss during stress, dose-dependently reversed sustained body weight loss of stressed animals. At Day 21 after stress, the body weight of stressed animals with high-dose pretreatment completely returned to normal levels. Treatment with MDL 11,939 after stress, on the other hand, was without effect on body weight and the animals with this treatment still experienced the long-term body weight loss. These findings suggested that there is an association between a decrease in the hypothalamic 5-HT_{2A} receptors and sustained body weight loss in stressed animals. The possible role of the hypothalamic 5-HT_{2A} receptor in mediation of feeding and body weight is discussed.

INTRODUCTION

Body weight loss is a characteristic response to repeated stresses, such as restraint and shock in rodents (Ottenweller et al., 1989;Harris et al., 2002;Smagin et al., 1999;Ottenweller et al., 1992;Dess et al., 1988;Donohoe et al., 1987). The stressed subjects, compared to nonstress controls, not only experience weight loss during or immediately after stress, but also demonstrate reduced body weight long after the cessation of the stressful episodes (Chotiwat and Harris, 2006;Harris et al., 2006;Ottenweller et al., 1992;Smagin et al., 1999). The initial weight loss during or immediately after stress has been shown to be due to reduced food intake and increased energy expenditure resulting from the activation of hypothalamic-pituitary-adrenal (HPA) axis. However, the sustained body weight loss in stressed subjects appears to involve currently unknown mechanisms that are distinct from the factors that cause initial weight loss (Harris et al., 2006;Smagin et al., 1999).

Since repeated stress can induce dysregulation of the serotonergic system (Maier and Watkins, 2005;Minor and Hunter, 2002), a brain aminergic system critically involved in regulating feeding and body weight (Heisler et al., 2003; Simansky, 1996b), it is likely that stress-induced sustained body weight loss is associated with dysregulation of the serotonergic system. The brain serotonergic system primarily interacts with the hypothalamus to regulate energy homeostasis. The serotonergic system in the medial hypothalamus, especially in the paraventricular nucleus (PVN), appears to act as a negative feedback mechanism to control feeding and body weight (Leibowitz and Alexander, 1998;Tachibana et al., 2001). Alterations of the hypothalamic serotonergic system are closely associated with abnormal feeding behaviors and abnormal body

weight (Huang et al., 2004b;Huang et al., 2004a;Simansky, 1996;Wolfe et al., 1997;Bouwknecht et al., 2001). Given that the hypothalamic serotonergic system is subject to change after stress (Dwivedi et al., 2005;Petty et al., 1997;Wu et al., 1999;Edwards et al., 1991), there may be an association between alterations in the hypothalamic serotonergic system and stress-induced sustained weight loss.

Among the hypothalamic serotonin receptors, the 5-HT_{1B} receptor and 5-HT_{2C} receptor appear to be the primary serotonin receptors involved in the serotonergic regulation of feeding and body weight (De Vry and Schreiber, 2000;Heisler et al., 2003;Simansky, 1996). Stimulation of hypothalamic 5-HT_{1B} or 5-HT_{2C} receptors leads to a hypophagic effect by accelerating satiety processes. Recent evidence suggested that hypothalamic 5-HT_{1A} and 5-HT_{2A} receptors may also participate in the serotonergic regulation of feeding and body weight (Collin et al., 2002;Fletcher and Coscina, 1993;Mancilla-Diaz et al., 2005;Simansky, 1996). In particular, the hypothalamic 5-HT_{2A} receptor may be involved in stress-related body weight change (Rosmond et al., 2002b;Rosmond et al., 2002a) and long-term regulation of energetic homoeostasis. Thus, a high level of the hypothalamic 5-HT_{2A} receptors is always associated with obesity (Nonogaki et al., 2006;Huang et al., 2004b;Park et al., 1999;Holmes et al., 2002;Li et al., 2003), while a low level of the hypothalamic 5-HT_{2A} receptor is associated with severe weight loss (Bailer et al., 2004;Kaye et al., 2005;Kaye et al., 2001). Taken together, there are multiple hypothalamic serotonin receptors participating in the mediation of energy homeostasis and body weight.

Thus, in order to examine whether there is an association between alterations in the hypothalamic serotonergic system and stress-induced sustained weight loss, the

present study first examined the effect of repeated restraint-tail shock stress on gene expression of the hypothalamic 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptors. Our results demonstrated that restraint-tail shock stress significantly downregulated mRNA levels of the hypothalamic 5-HT_{2A} receptor and upregulated 5-HT_{1B} receptor mRNA levels, while mRNA levels of 5-HT_{1A} and 5-HT_{2C} receptors remained unchanged. Furthermore, pretreatment with a specific 5-HT_{2A} receptor antagonist reversed stress-induced long-lasting low body weight in stressed subjects. These findings suggested that the hypothalamic 5-HT_{2A} receptor plays a critical role in mediating stress-induced sustained body weight loss, and that the 5-HT_{2A} receptor antagonists might have potential in regulating body weight loss in stress-associated psychiatric disorders, such as depression and PTSD.

METHODS

Stress protocol and body weight measurement

All animal experiments were performed in accordance with our institutional guidelines after obtaining the approval of the Institutional Animal Care and Use Committee (IACUC). A total of 40 male Sprague-Dawley rats initially weighing between 100 and 150 g (Taconic Farms, Germantown, NY, USA) were used in the study. Animals were housed in a climate controlled environment in groups of two per cage with free access to food and water, and were maintained on a reverse light/dark cycle (light on 18:00) at 22 $^{\circ}$C. The animals were equally assigned to five groups based on their body weight. The five groups include a control group; a stress + vehicle group, in which animals received 0.01N HCl as vehicle before or after they underwent the stress protocol each day for three consecutive days; a pre-stress low-dose group, in which animals received 0.5 mg/kg 5-HT_{2A} receptor antagonist MDL 11,939 (Tocris Bioscience, Ellisville, Missouri) 30 minutes before stress each day for three consecutive days; a pre-stress high-dose group, in which animals received 1.5 mg/kg MDL 11,939 30 minutes before stress each day for three consecutive days; and a post-stress group, in which animals received 1.5 mg/kg MDL 11,939 immediately after stress each day for three consecutive days. The drugs or vehicle were delivered by intraperitoneal injection (1ml latex free syringe w/ 27G½ needle, B-D, Franklin Lakes, NJ).

Stress exposure consisted of a 2-h per day session of immobilization and tail-shocks, for 3 consecutive days. The animals were stressed in the morning (between 0800 and 1200). They were restrained in a plexiglas tube, and 40 electric shocks (1 mA, 3 s duration) were applied at varying intervals (140–180 s). This stress protocol was adapted

from the ‘learned helplessness’ paradigm in which animals undergo an aversive experience under conditions in which they can not perform any adaptive response (Seligman and Maier, 1967;Seligman and Beagley, 1975). The rats were stressed for three consecutive days because it has been previously demonstrated that repeated stress sessions for three days is more effective than a single stress session in producing physiological and behavioral abnormalities associated with depression and anxiety disorders (Ottenweller et al., 1989;Servatius et al., 1995). More stress sessions, beyond three days, does not appear to produce greater physiological and behavioral changes (Ottenweller et al., 1992;Ottenweller et al., 1989).

At the day of stress, all animals were weighed before stress both as a physiological measure and as a metric for balancing the groups. After exposure to stress, animals were weighed immediately after the stress, at day 7, day 10, day 12, day 21 and day 30 poststress. For each test day, ANOVAs for measures were performed on net weight gain with the factors of stress status and drug dosage. The data were represented as mean±S.E.M.

Quantitative real-time PCR

The hypothalamus was dissected and frozen. Frozen tissues of 30 mg were homogenized and total RNA was extracted using the RNeasy kit (Qiagen, Germany) following the manufacturer’s protocol. One microgram (1 μg) of total RNA was reverse transcribed into first-strand cDNA using the RETROscript reverse transcriptase kit and oligo dT primers (Ambion Inc., Austin, TX) according to the manufacturer’s protocols. One microliter of cDNA from the RT-reaction was used as the template for quantitative

real-time PCR reaction with a final PCR reaction volume of 25 µl, with the 5' and 3' gene specific PCR primer concentrations at 10 pM each. PCR primers were designed using Primer3 software (Whitehead Institute, MIT, MA) and according to the coding sequences of each gene (GenBank#: 5HT1A: #J05276; 5HT1B: #X62944; 5HT2A, #X13971; 5-HT2C, # NM_012765; Table 1). Quantification of mRNA expression was performed (in triplicate) using the SYBR Green SuperMix (BioRad, CA) and a 2-step PCR reaction procedure, performed on the MyiQ Single Color Real-Time PCR Detection System (BioRad, CA). In brief, after the initial denaturation at 95^{o}C for 3 min, 45 cycles of primer annealing and elongation were conducted at 60^{o}C for 45 seconds, followed by denaturation at 95^{o}C for 10s. Fluorescent emission data were captured, and mRNA levels were quantified using the threshold cycle value (Ct). To compensate for variations in input RNA amounts and efficiency of reverse transcription, data for 5-HT_{1A}, 5-$HT_{1B,}$ 5-HT_{2A} and 5-HT_{2C} receptor mRNA for each sample were normalized by reference to the data obtained for the house keeping β-actin gene (GenBank accession #. BC063166) determined from the same sample. Fold change in mRNA expression was calculated using the equation: fold change=$2^{-\Delta\Delta Ct,}$ where ΔCt=target gene Ct-house keeping gene (β-actin) Ct, and ΔΔCt is ΔCt control - ΔCt stress (or fold change) =$2^{\wedge(\Delta CT\ control\ -\ \Delta CT\ stress)}$. The mean and S.E.M. were calculated from three replicate amplifications. Each RT-PCR assay was repeated twice.

Differences between the stress and control groups were examined for statistical significance using one-way ANOVA (with stress as the main factor) analysis followed by post-hoc Fisher's test. A difference with *P*-value less than 0.05 was considered statistically significant.

RESULTS

1. The effects of stress on gene expression of the hypothalamic 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptor.

As illustrated in Figure 1A, C, exposure to three-day stress caused a significant decrease in the mRNA levels of the hypothalamic 5-HT_{2A} receptors ($n=8$, $p \leq 0.01$), and an increase in the mRNA levels of the hypothalamic 5-HT_{1B} receptors ($n=8$, $p \leq 0.01$), compared with control rats. The mRNA levels of the hypothalamic 5-HT_{1A} receptors and 5-HT_{2C} receptor was not significantly changed by stress ($n=8$, $P>0.05$ for both serotonin receptor types) (Figure 1B, 1D).

2. Pretreatment with the 5-HT_{2A} receptor antagonist MDL 11,939 reversed stress-induced sustained weight loss.

As illustrated by Figure 2A, three days of inescapable tail-shock significantly decreased body weight [$F(1, 38) = 65.329$, $p<0.001$]. This effect was still observed even 30 days after stress (Fig. 2A). Pretreatment with MDL 11,939 appeared to exacerbate the initial body weight loss in a dose-dependant manner, although not significantly [$F(2,21) = 1.726$], compared to stressed animals with vehicle treatment (Fig. 2A,2B). However, pretreatment with MDL 11,939 dose-dependently increased the body weight of stressed animals from Day 7 after stress to the end of the observation period, compared to vehicle control (Day 30 after stress, Fig.2A, 2B). On Day 12 after stress, the animals that received high-dose pretreatment gained significantly more weight than the animals that received vehicle treatment (104.5 ±3.8g and 93.8± 3.5g respectively; $p<0.05$). On Day 21 after stress (Fig.2A, 2B), the body weight of stressed animals that received high-dose

pretreatment was almost equal to the control animals (weight gain: 173.0±4.0g and 174.0±5.3g respectively), which is significantly different from the group of stressed animals that received vehicle treatment (156.8±4.1g, $p<0.05$). The effect still remained on Day 30 (209.1 ±8.0 and 208.9±9.2 for the control and high-dose pretreatment groups, respectively). Interestingly, MDL 11939 treatment after stress was not able to increase the body weight of the stress animals during one-month observation period. The weight gain of this group of animals was not significantly different from the vehicle-treated group of stressed animals (Fig. 2A, 2B, $p>0.05$ at all time points).

DISCUSSION

The effect of stress on gene expression of the hypothalamic 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptors.

The data demonstrated that stress differentially altered gene expression of the hypothalamic 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptor. Stress decreased gene expression of the 5-HT_{2A} receptor, increased the 5-HT_{1B} receptor gene expression, while the 5-HT_{1A} receptor and 5-HT_{2C} receptor were unchanged. Indeed, a similar mRNA change pattern of the hypothalamic 5-HT_{2A}, 5-HT_{2C}, 5-HT_{1A} receptors after stress has also been reported previously (Dwivedi et al., 2005;Petty et al., 1992). Furthermore, the protein levels of these receptors were also subject to a similar change as their mRNAs after stress (Dwivedi et al., 2005;Petty et al., 1997;Wu et al., 1999). These observations indicate that the hypothalamic 5-HT_{2A} receptors could be readily downregulated by different types of stress, while the hypothalamic 5-HT_{1A} and 5-HT_{2C} receptors are resistant to modification by stress. Although the hypothalamic 5-HT_{1A} and 5-HT_{2C} receptors are involved in the serotonergic regulation of feeding and body weight, no change of these two receptors after stress suggests that these two receptors may not participate in the occurrence of stress-induced physiological change, such as sustained weight loss.

Unexpectedly, our study observed that stress could upregulate the hypothalamic 5-HT_{1B} receptor mRNA. If the level of mRNA corresponds to the capacity of a cell to synthesize the mature protein, the hypothalamic 5-HT_{1B} receptors would be upregulated. However, a previous study, using a similar stress protocol, demonstrated that stress could result in a decrease of 5-HT_{1B} receptor binding in the hypothalamus (Edwards et al.,

1991). Since the stress protocol used in their studies is not exactly same as the protocol used in present study, this seeming difference in the change of the 5-HT_{1B} receptor may be due to the difference in the stress protocols. Alternatively, this difference in the change of the mRNA and protein of the 5-HT_{1B} receptor may reflect the fact that the protein and mRNA was not always changed in the same direction (Gray and Roth, 2001). Examining the change in the 5-HT_{1B} receptor protein levels after stress may be able to unravel the reason for this difference.

Although the mechanisms underlying stress-induced 5-HT_{1B} receptor change is not well documented, downregulation of 5-HT_{2A} receptors after inescapable stress is relatively clear in the literature. Inescapable shock stress can enhance serotonin levels in the hypothalamus, and this enhancement could last long after the end of stress (Maier and Watkins, 2005;Tsuchiya et al., 1996;Shimizu et al., 1992;Vermes and Telegdy, 1977;Torres et al., 2002;Shimizu et al., 1989). Since the 5-HT_{2A} receptor is primarily a postsynaptic receptor, it is believed that prolonged stimulation of the 5-HT_{2A} receptor by excess hypothalamic 5-HT level results in adaptation of this receptor (i.e., receptor desensitization, internalization and downregulation). Indeed, receptor downregulation after chronic agonist stimulation is a common phenomena for the 5-HT_{2A} receptor, and it has been observed in both culture (Roth et al., 1995) and in *in vivo* systems (Damjanoska et al., 2004;Anji et al., 2000;Smith et al., 1999). Interestingly, the 5-HT_{2C} receptor, although coupled to the similar signal pathway as the 5-HT_{2A} receptor, appears to be resistant to chronic agonist stimulation (Smith et al., 1999). This may explain the observation that stress only decreased the mRNA level of the hypothalamic 5-HT_{2A} receptor without affecting the 5-HT_{2C} receptor.

5-HT_{2A} receptor blockade before stress normalized body weight in stressed animals.

Since the hypothalamic 5-HT_{2A} receptor and 5-HT_{1B} receptor are critically involved in feeding and body weight regulation (see Introduction), and these two receptors were changed after stress, it is likely that alterations of these two receptor signaling are associated with stress-induced sustained body weight loss. To further examine this association, we treated the rats with a selective 5-HT_{2A} receptor antagonist, MDL 11,939, before stress or after stress. Since decreased gene expression of the hypothalamic 5-HT_{2A} receptor should result from prolonged stimulation by stress-elevated 5-HT, and antagonists can prevent agonist-induced downregulation of receptor gene expression (Brown et al., 1998;Bieck et al., 1992;Baumhaker et al., 1993;Horackova et al., 1990;Lassegue et al., 1995;Scarceriaux et al., 1996;Chau et al., 1994;Fukamauchi et al., 1993), MDL 11,939 pretreatment would prevent this receptor gene expression from being downregulated by stress. Considering that the 5-HT_{1B} receptor, in addition to being a postsynaptic receptor in the hypothalamus, is also a presynaptic receptor localized at the raphe nuclei and serotonergic terminals (Olivier and van Oorschot, 2005;Sari, 2004), and any 5-HT_{1B} receptor ligands would affect the dorsal raphe 5-HTergic system *per se*, 5-HT_{1B} receptor ligands thereby were not chosen in this experiment strategy.

We found that rats exposed to three-day restrain-tail shock stress experienced a significant weight loss immediately after stress and did not return to the weight of their non-stress controls for at least 30 days after stress. In fact, such a phenomena has been repeatedly observed previously with a much milder stress protocols (Chotiwat and Harris, 2006;Harris et al., 2002;Harris et al., 2006;Smagin et al., 1999), and the difference in weight between stress and control animals could be maintained for as long as 11 weeks

after the restraint stress (Harris et al., 2006). As shown in Figure 2A, the stressed rats appeared to start to gain weight at the same rate as their non-stressed controls when stress had ended, but it appears that they have no attempt to overeat to compensate for the energy deficit experienced during stress and immediately after stress. This observation is also compatible with the previous report showing that once stress ended, there is no difference in food intake and energy expenditure between stress and control animals. Although food intake and energy expenditure were not measured in our current study, the same rate in weight gain between stress and control groups in our study indicates that the animals experiencing the traumatic stress as used in the present study may also have the same food intake as control after stress has ended.

MDL 11,939 administrated prior to stress appears to dose-dependently exacerbate initial stress-induced weight loss, although not significantly. Since the effect of MDL 11,939 at this point (immediately after stress) results from 2-dosages other than 3 dosages, measuring at day 1 after stress (3 dosages) may be able to detect a significant effect of this compound (unfortunately we did not measure body weight at this point). The reducing effect of this compound on body weight is compatible with previous reports. In these studies, administration of the 5-HT_{2A} receptor antagonists at a very high concentration was able to reduce the feeding behaviors in rodents, suggesting that MDL 11, 939 in this study may reduce the body weight by further decreasing food intake in the stressed animals. However, the administration of this compound immediately after stress did not tend to exacerbate initial stress-induced weight loss (Figure 2B). In addition, certain studies also suggested that activation of the 5-HT_{2A} receptor leads to a disruption of the feeding cascade (De Vry J. and Schreiber, 2000). These observations suggested

that the 5-HT_{2A} receptor may have a limited, if any, role in mediating short-term feeding behaviors.

Although MDL 11, 939 pretreatment tended to exacerbate stress-induced initial weight loss, this pretreatment could reverse sustained reduction of body weight of stressed animals. By Day 7 after stress, the animals with pretreatment tended to gain more weight than stressed animals with vehicle treatment and posttreatment of this antagonist (weight gain: 61.12g and 60g versus 56.87g and 55.88g, respectively). On Day 12 after stress, this tendency became significant. On Day 21 after stress, the body weight of stressed animals with high-dose pretreatment is almost equal to nonstress controls. These observations suggested that in stressed animals with the pretreatment, their bodies appear to be able to detect a lower body weight than normal and overeat to compensate the energy deficit during and immediately after stress. Interestingly, the treatment immediately after stress, not only was without effect on initial weight loss during the stress, but also was not able to normalize the body weight. The stressed subjects with post-stress treatment still experienced the sustained body weight loss as observed in stressed animals with vehicle treatment. This suggests that the pathophysiology associated with sustained weight loss could be developed after the first stressful episodes. After the first stressful episodes, it is too late to interfere with the development of these pathophysiological changes.

The role of the hypothalamic 5-HT_{2A} receptor in regulation of body weight.

The present findings that stress decreased gene expression of the hypothalamic 5-HT_{2A} receptor, and pretreatment with the 5-HT_{2A} receptor antagonist MDL11,939

reversed the sustained weight loss of stressed animals, suggest that there may be association between the decrease of the hypothalamic 5-HT_{2A} receptor and sustained low body weight in stressed animals. As mentioned before, downregulation of the hypothalamic 5-HT_{2A} receptor after stress has also been observed previously (Dwivedi et al., 2005;Petty et al., 1997;Wu et al., 1999). Furthermore, the decrease of this hypothalamic receptor is long-term and is associated with behavioral depression (Dwivedi et al., 2005). As stated in the introduction, the hypothalamic 5-HT_{2A} receptors may be involved in regulation of long-term energetic homeostasis. The level of hypothalamic 5-HT_{2A} receptor appears to be positively related to body mass and genetic variation of 5-HT_{2A} receptor could result in the abdominal obesity in human (Rosmond et al., 2002b;Rosmond et al., 2002a). These observations suggest that the hypothalamic 5-HT_{2A} receptor may be an important component of "set-point" in regulation of body weight (Harris, 1990). The high or low level of the hypothalamic 5-HT_{2A} receptor may determine an individual's final body weight. Repeated stress decreases this hypothalamic serotonin receptor, thus, lowers the set-point and results in a low body weight in stressed animals. The pretreatment of MDL 11,939, which is assumed to prevent this receptor from being long-term downregulated, may maintain this receptor intact (set-point not changed). This may allow stressed animals detect a lower body weight than normal and overeat to meet the balance determined by set-point. MDL 11, 939 treatment after stress may be too late to prevent set-point resetting by stress, and these animals thereby have a lower set-point and a low body weight.

A previous study has shown that blockade of corticotrophin-releasing factor (CRF) signaling before stress could prevent the stress-induced initial weight loss and

sustained weight loss (Smagin et al., 1999). However, it appears that the sustained weight loss is not associated with the chronic change of hypothalamic CRF systems, but involves other cascade mechanisms initiated by CRF (Harris et al., 2006;Smagin et al., 1999). The hypothalamic 5-HT_{2A} receptor downregulation is very likely such a CRF-initiated cascade mechanism responsible for sustained body weight loss. As mentioned before, the hypothalamic 5-HT_{2A} receptor downregulation is very likely due to prolonged stimulation of this receptor by stress-enhanced hypothalamic 5-HT levels, while the stress-induced enhancement of hypothalamic 5-HT is believed to be mediated by CRF (Maier and Watkins, 2005;Staub et al., 2006;Staub et al., 2005;Pernar et al., 2004;Lowry et al., 2000;Li et al., 1998). Hence, elevated CRF during repeated restrain stress, by enhancing the hypothalamic serotonin level, may impair the hypothalamic 5-HT_{2A} receptor signaling. Impaired 5-HT_{2A} receptor signaling in the hypothalamus may be responsible for a low body weight in stressed animals.

Other possible mechanisms responsible for a low body weight of stressed animals

Previous studies have demonstrated that the same stress protocol as used in the present study appears to induce persistent elevated basal corticosterone in stressed subjects (Ottenweller et al., 1989;Ottenweller et al., 1994;Ottenweller et al., 1992). This chronic activation of the hypothalamic-pituitary-adrenal (HPA) axis may be responsible for sustained body weight loss in stressed subjects (Servatius et al., 1995). Previous studies have shown that the hypothalamic 5-HT_{2A} receptor is involved in stress-induced release of CRF from the paraventricular nucleus (PVN) (Zhang et al., 2002;Mikkelsen et al., 2004;Hanley and Van de Kar, 2003;Van de Kar et al., 2001;Saphier et al., 1995).

MDL 11,939 pretreatment may block 5-HT_{2A} receptors in the PVN, and thereby interfere with the development of stress-induced chronic status of the HPA axis. The animals thereby maintains a normal HPA axis, which allows them have a normal body weight. However, the subjects exposed to much milder stress (restraint only) did not show the evidence of chronic activation of HPA axis after stress, although they experienced a similar sustained body weight loss as observed in the present study (Harris et al., 2006;Harris et al., 2002;Smagin et al., 1999). Hence, the change of the HPA axis may not be a critical factor responsible for the occurrence of a low body weight in stressed subjects.

Stress could upregulate the gene expression of the hypothalamic 5-HT_{1B} receptors, suggesting alterations in this receptor may be involved in the occurrence of a lower body weight in stressed animals than control. The hypothalamic 5-HT_{1B} receptor also is a primary serotonin receptor involved in feeding and body weight regulation. Stimulation of hypothalamic 5-HT_{1B} receptors accelerate satiety processes during feeding behaviors (De Vry and Schreiber, 2000;Heisler et al., 2003;Simansky, 1996). Knockout of this receptor, thus, results in obesity in mice (Lee et al., 2004;Bouwknecht et al., 2001). Also, increased levels of this receptor in the hypothalamus has been associated with anorexia and sustained weight loss (Makarenko et al., 2005b;Makarenko et al., 2005a). Hence, alteration of this hypothalamic serotonin receptor after stress, in addition to alterations of 5-HT_{2A} receptors, may also participate in the occurrence of a low body weight in stressed subjects.

Functional implications.

Since repeated restraint stress has long been adopted as an animal model of depressive illness and anxiety disorders (Maier and Watkins, 2005;Minor and Hunter, 2002;Servatius et al., 1995), sustained body weight loss resulting from this stress may mimic weigh loss observed in patients with depression and anxiety disorders, such as PTSD (Evers and Marin, 2002;Hopkinson, 1981;Myers et al., 2005;Sutker et al., 1990). The present finding that pretreatment of the 5-HT_{2A} receptor antagonist could reverse the sustained body weight loss in stressed animals, in the context that this hypothalamic receptor could be downregulated by stress, suggests that alteration of the hypothalamic 5-HT_{2A} receptor signaling plays an important role in occurrence of stress-induced sustained body weight loss. This finding may have implications in medical intervention of weight loss observed in stress-associated psychiatry disorders, such as depression and PTSD.

Figure 1

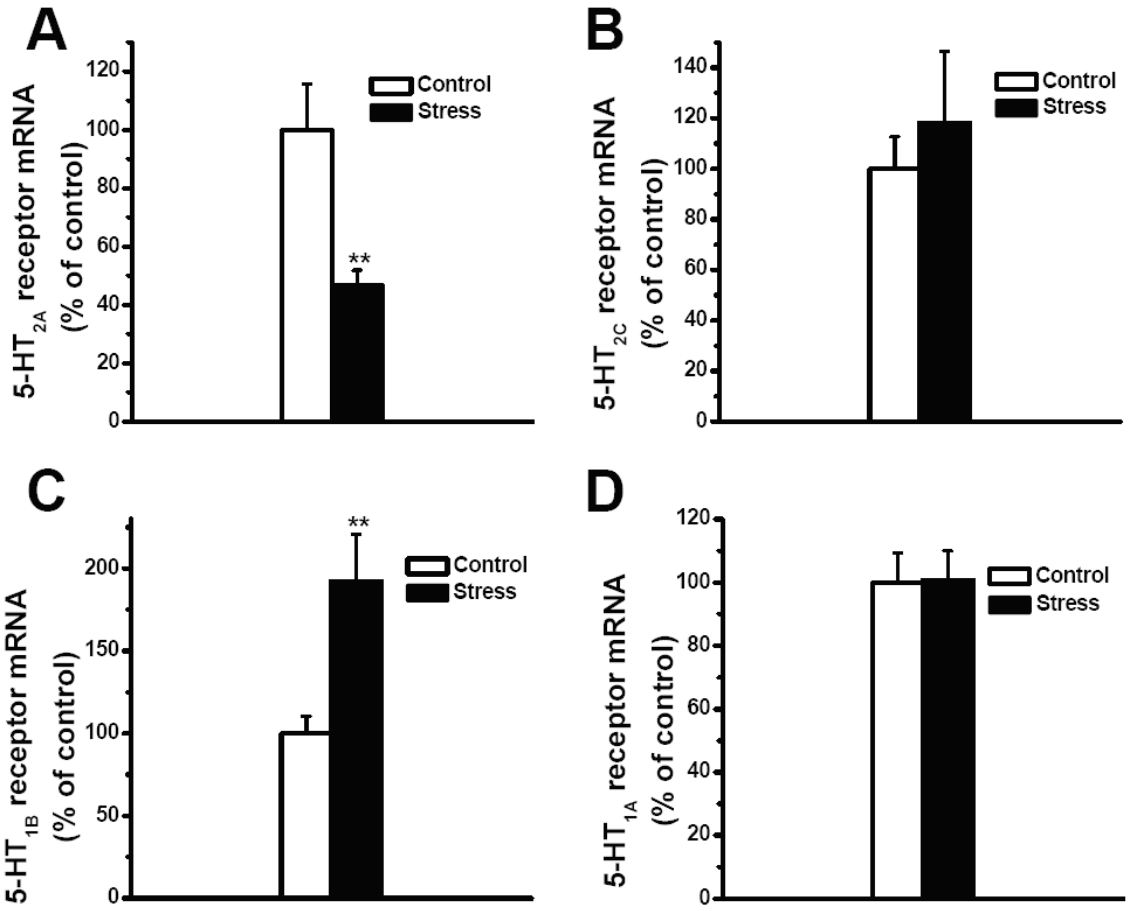
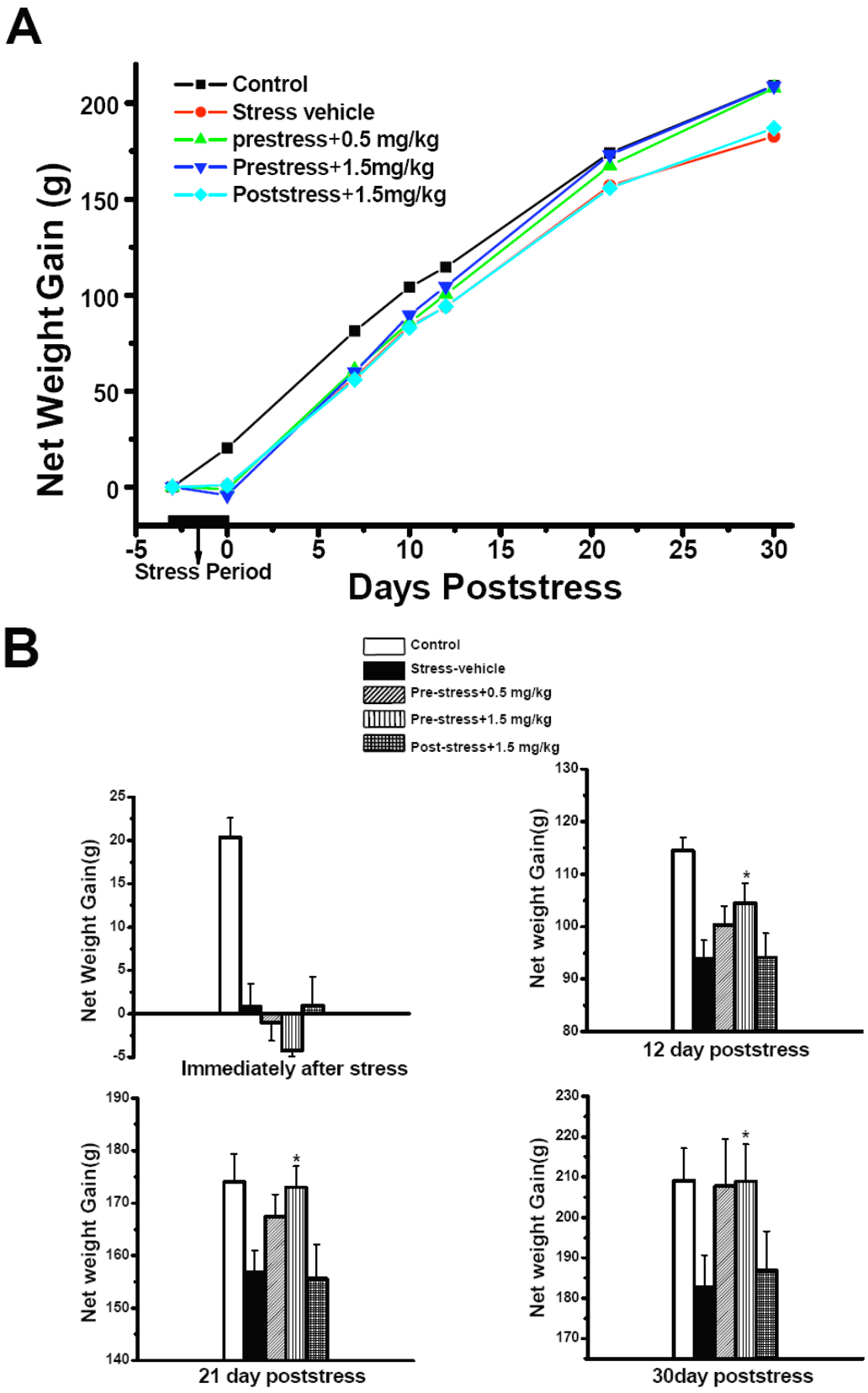


Figure 1. Stress significantly decreased the expression of the hypothalamic 5-HT_{2A} receptor mRNA (n = 8, p≤ 0.01) (**A**), and increased expression of 5-HT_{1B} receptor mRNA (n=8, p≤ 0.01) (**C**). The mRNA level of the hypothalamic 5-HT_{1A} receptor (**D**) and 5-HT_{2C} receptor (**B**) is not significantly changed by stress (n=8, p>0.05 for both receptor types).

Figure 2. The effect of MDL 11, 939 on weight gain after the stress. Mean of net body weight gain of male Sprague–Dawley rats in different groups are plot against the days after stress (**A**). The body weight was measured on the day immediately after stress, 7, 10, 12, 21, 30 days after stress. (**B**). Means ± S.E.M of net weight gains of all groups at immediately after stress, 12, 21, and, 30 days after stress. Asterisks indicate that there was a significant ($P \leq 0.05$) difference in body weight between this group and the group with vehicle treatment.

Figure 2


A
Control
Stress vehicle
prestress+0.5 mg/kg
Prestress+1.5mg/kg
Poststress+1.5mg/kg
Net Weight Gain (g)
Stress Period
Days Poststress
B
Control
Stress-vehicle
Pre-stress+0.5 mg/kg
Pre-stress+1.5 mg/kg
Post-stress+1.5 mg/kg
Net Weight Gain(g)
Immediately after stress
Net weight Gain(g)
12 day poststress
21 day poststress
30day poststress

CHAPTER 4

HISTAMINERGIC MODULATION OF EXCITATORY SYNAPTIC TRANSMISSION IN THE RAT BASOLATERAL AMYGDALA

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ABSTRACT

The effects of histamine on excitatory synaptic transmission between the external capsule and basolateral amygdala (BLA) were examined using intracellular and field potential recordings in rat amygdala slices. Bath application of histamine (20 μM) suppressed intracellular excitatory postsynaptic potentials (EPSPs; 70.3±5.1% of control amplitude) in 43 of 64 BLA neurons, and potentiated EPSPs (341±81% of control amplitude) in 21 neurons, without changing resting membrane potential or input resistance. The histamine-induced suppression of EPSPs was accompanied by an increase in paired-pulse facilitation of the slopes of EPSPs, suggesting a presynaptic locus of the action. The suppressive effect could be blocked by the selective H_3 antagonist thioperamide, and mimicked by the selective H_3 agonist *R*-α-methylhistamine, indicating that the suppressive effect is mediated by the presynaptic H_3 receptor. The potentiating effect of histamine on EPSPs was not accompanied by the change of paired-pulse facilitation and was not affected by the presence of H_1, H_2 or H_3 receptor antagonists. In addition, the effective concentration of agonist to produce 50% of maximal response (EC50) of the potentiating action of histamine is 49 nM, much lower than the EC50 (470 nM) of the H_3 receptor-mediated suppressive effect characterized here. These observations suggest a novel, high affinity and postsynaptically mediated effect of histamine. In extracellular recordings, histamine, at low concentration (200 nM), consistently potentiated field potentials. At high concentration (20 μM), histamine suppressed field potentials, but potentiated field potentials when H_3 receptors were blocked. Taken together, these results revealed that histamine, via the presynaptic H_3 receptor and a currently unknown mechanism, decreases or increases excitatory synaptic

transmission in the BLA respectively. This specific histaminergic modulation of neuronal activity in the amygdala may play an important role in amygdala-mediated physiological and pathophysiological processes, such as fear, emotional learning and memory, temporal lobe epilepsy, and affective disorders.

INTRODUCTION

The amygdala is activated during stressful and emotional experiences via cortical and subcortical afferents to the lateral and basolateral amygdaloid nuclei (BLA)(Braga et al., 2004;Aggleton JP and Sauder RC, 2000). In addition to glutamatergic afferents, the BLA also receives dense input from modulatory aminergic systems originating in the hypothalamus and brain stem. The histaminergic fibers, exclusively arising from the tuberomammillary nucleus (TMN) of the hypothalamus (Panula et al., 1984;Brown et al., 2001;Haas and Panula, 2003), densely innervate the amygdala complex, and histamine receptors are heavily expressed in the BLA (Pollard et al., 1993;Ryu et al., 1995b). However, the role of histamine in modulating neuronal excitability and synaptic transmission in the amygdala has not been investigated.

The modulatory actions of histamine in the brain, known so far, occur through at least three G-protein coupled receptor subtypes. Histamine H_1 and H_2 receptors are postsynaptic receptors, which are coupled to activation of phospholipase C and adenylyl cyclase, respectively (Schwartz et al., 1991b). Histamine H_3 receptors are autoreceptors or heteroreceptors, regulating the release of histamine and other neurotransmitters (Arrang et al., 1983;Arrang et al., 1985;Brown et al., 2001). Autoradiographic studies have demonstrated that histamine H_3 receptors are intensively expressed in the central, lateral and basolateral nuclei of the amygdala (Pollard et al., 1993;Ryu et al., 1995b), while H_1 and H_2 receptor binding is relatively low in these major amygdala nuclei, in either rat or human brain (Ryu et al., 1995b;Honrubia et al., 2000;Karlstedt et al., 2001). Histamine, when injected directly into the amygdaloid complex, suppresses kindling (Wada et al., 1996;Kamei et al., 1998;Kamei, 2001a) and impairs aversive conditioned

learning in rats (Alvarez and Ruarte, 2002). The histamine receptor subtypes mediating these inhibitory effects of histamine in the amygdala are still unclear. In addition, activation of H_3 receptors by injection of selective H_3 receptor agonists in the basolateral amygdala (BLA) improved expression of fear memory (Passani et al., 2001;Cangioli et al., 2002). The mechanism underlying such a facilitating action of H_3 receptor agonists in the amygdala-mediated behavior also remains to be understood.

Using an amygdala slice preparation and available pharmacological tools, the present study examined the modulatory actions of the histaminergic system on neuronal excitability and synaptic transmission in the BLA. Our results demonstrated, for the first time in the BLA, that histamine depresses excitatory synaptic transmission through a presynaptically located H_3 receptor. Moreover, a facilitating effect of histamine on AMPA/kainate receptor mediated synaptic transmission was observed in a portion of BLA neurons. This facilitating effect on synaptic transmission in the BLA appears to be unrelated to currently known histamine receptors.

MATERIALS AND METHOD

Amygdala slice preparation.

Male Sprague-Dawleyrats weighing 75–150 g (4-6 weeks) were decapitated after light anesthesia with halothane, the brains were rapidly removed, and 450-µm-thick transverse slices of the amygdala were cut from tissue blocks with a Vibratome (Technical Products International, St. Louis, Missouri). The slices were preincubated in artificial cerebrospinal fluid (ACSF), continuously bubbled at room temperature (23°C) with 95% O_2 /5% CO_2 to maintain a pH of 7.4, for at least 1 hour before use. The ACSF contained 117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$ and 11 mM glucose.

Intracellular and field potential recording.

Slices were transferred to an interface chamber that was continually superfused with ACSF at a rate of 1–2 ml/min. The temperature of perfusion solution in the chamber was maintained at 32 °C through a TC-202A Bipolar temperature controller (Harvard Apparatus, Inc., Holliston, Ma, USA). Microelectrodes were pulled from microfiber-filled borosilicate capillaries (OD 1.0 mm, ID 0.58 mm for intracellular recording; OD 1.0 mm, ID 0.78 mm for extracellular recording) using a Flaming-Brown horizontal micropipette puller (Sutter Instruments, Novato, CA, USA). The resistance of the intracellular microelectrodes filled with 3 M KCl ranged from 80 to 130 MΩ, while the resistance of extracellular recording electrodes filled with 3 M NaCl ranged from 2 to 5 MΩ. The microelectrode tips were visually positioned in the basolateral region of the amygdala (between the external capsule (EC) and the bed nucleus of the stria terminalis)

using a dissecting microscope (see Fig.1). Intracellular impalements were made in a blind fashion and recordings were made from pyramidal neurons. The neurons were identified as the pyramidal cells on the basis of their accommodation response and by a prominent afterhyperpolarization that followed a current evoked burst of action potentials (Washburn and Moises, 1992). Intracellular recordings were terminated if the resting membrane potential was less negative than -55 mV, or if the action potential height was less than 70 mV. Intracellular potential was amplified with an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA, USA; low-pass filter, 3 kHz). Field potentials were amplified with a differential amplifier (Warner Instrument Corporation, Hamden, CT, USA). The output was digitized with a Digidata 1200 interface (Axon Instruments, Foster City, CA, USA). On- and off-line data acquisition and analysis was carried out using the Whole Cell Electrophysiology Program (WCP) version 1.7b (John Dempster, University of Strathclyde, Glasgow, UK).

Stimulation

Synaptic responses were evoked with sharpened tungsten bipolar stimulating electrodes (World Precision Instruments, Sarasota, Florida) placed in the EC (see Fig.1). The stimulating electrode was ~2 mm from the recording site. Stimuli were delivered using photoelectric stimulus isolation units having a constant current output (ISO-Flex; Stimulus Isolation Unit, Jerusalem, Israel). The stimulus intensity was adjusted to produce a synaptic response 30–50% of maximum amplitude without triggering an action potential response. Peak response amplitudes were measured with respect to the resting membrane potential. Single 0.1ms monophasic square pulses were applied continuously

throughout the experiment at 0.1 Hz. Such stimulation was associated with no more than a 10% drift in the peak synaptic response amplitude during experiments lasting up to 120 min.

Drug application

Chemicals and drugs included (–)-Bicucculline methochloride, D-2-amino-5-phosphonovalerate (D-APV), SCH50911 [(+)-(2S)-5,5-dimethyl-2-morpholin-eacetic acid], R-alpha-methylhistamine dihydrobromide, mepyramine maleate (2-((2-dimethylamino) ethyl)(p-methoxybenzyl) amino)-pyridine maleate), cimetidine, thioperamide, triprolidine, clobenpropit and tiotidine were from Tocris Cookson (Ellisville, Missouri). Histamine was obtained from Sigma (St.Louis, Missouri). Ly293558 was a generous gift of Eli Lilly Research Laboratories (Indianapolis, IN). Drugs were dissolved in distilled water or Dimethyl Sulfoxide (DMSO, Fisher Biotech), neutralized and stored as stock solutions, 1000 times the required concentration, until use. The stock solution was kept for no more than 10 days. During the experiments the drugs were applied to the perfusion solution at desired concentrations.

Data are expressed as mean±S.E.M. Statistical comparisons were made with Student's unpaired or paired *t*-test, as appropriate.

RESULTS

1. Effects of histamine on excitatory postsynaptic potentials (EPSPs) in the BLA

The effects of 20 μM histamine on the membrane properties of BLA neurons were examined first. The resting membrane potential remained unaffected by the application of 20 μM histamine in the vast majority of the BLA neurons recorded (104/110, 94.6%) (Figure1 A1,B1). The mean resting potential of these neurons before and 15 min after the administration of 20 μM histamine was -68.7± 1.3 mV and -67.8±1.4 mV respectively (n=10, P>0.05). Only six out of 110 neurons (6/110, 5.4%) showed apparent depolarization of the membrane potential in response to administration of 20 μM histamine. These neurons were not included in subsequent studies and data analysis. The mean input resistances determined from the voltage response to 100 pA hyperpolarizing currents injected through the recording electrode were not significantly different before and 15 min after administration of histamine (Figure1A1, B1 and Table 1). The voltage responses and action-potential firing patterns evoked by depolarizing current steps were also monitored before and after the application of histamine. As illustrated in Figure 1A2, B2 and Table 1, the action potential firing patterns and the voltage responses obtained by injecting 200-600 pA, 250 ms current before, during and after the application of 20 μM histamine were not significantly altered in the BLA neurons. Thus, histamine does not significantly change the resting membrane potential and input resistance of the BLA neurons.

Next, we examined the actions of histamine on excitatory synaptic transmission, in the BLA. Intracellularly recorded excitatory postsynaptic potentials (EPSPs) evoked by electrical stimulation of the external capsule (EC) were isolated by inclusion of 10 μM

bicucculline, a $GABA_A$ receptor antagonist, and 5μM SCH 50911, a $GABA_B$ receptor antagonist, in the perfusion solution. At resting membrane potentials, the recorded EPSPs were predominantly mediated by AMPA/kainate receptors, as they were largely blocked by 20μM LY293558, an AMPA/kainate receptor antagonist (Figure 1A2 inset). Bath application of 20 μM histamine for 15 minutes produced a reduction in the amplitude of the EPSPs to 70.3±5.2% of baseline (n=20, $p<0.01$, Figure 1A1, 1A2 and 1A3), in 43 out of 64 neurons. The effect of histamine was evident between 3 to 5 minutes following initial drug application and the maximal effect was observed between 10 to 13 minutes following the histamine application. It was also observed that the amplitude of EPSPs began to gradually recover from histamine-induced suppression even while histamine remained in the perfusion solution. After 30 min of histamine washout, EPSPs recovered fully in 3 neurons, and partially in 40 neurons.

A striking potentiation effect of histamine on the amplitude of EPSPs (341±81% of baseline, n=16, $p<0.01$) was observed in 21 out of 64 neurons examined, without a significant change in the input resistance and resting membrane potentials as shown in figure 1B1, 1B2, 1B3 and table 1. In 18 of these 21 neurons, 20 μM histamine initially induced a transient depression of the EPSPs (85.6±6.9% of baseline) followed by a striking potentiation (Figure 1B3). In the remaining three neurons, 20 μM histamine directly induced potentiation of EPSPs without the preceding transient depressive effect. The maximal potentiation effect of histamine was observed 15-20 min after application of histamine. The potentiation effect of histamine, albeit smaller, remained 30 minutes after washout.

2. The suppression of EPSP is mediated by H_3 histamine receptor.

The dose-response relationship was investigated for the suppressive effect of histamine on intracellular EPSPs in BLA neurons. 10 μM histamine was sufficient to produce maximal suppression effect (70.4±3.9% of control levels, n=7, $P<0.01$. Figure2A), while 200 nM histamine did not produce a significant suppression (94.3±3.6% of control levels; n=6). The EC50 of histamine for this suppressive effect was 0.47 μM (Figure 2D).

The selective H_1, H_2 and H_3 receptor antagonists were used to determine the histamine receptor subtypes mediating the histamine-induced suppression of EPSPs. The presence of neither the H_1 receptor antagonist mepyramine maleate (5μM, n=12) nor the H_2 receptor antagonist cimetidine (50μM, n=13) in the perfusion solution could prevent the depressive effect of 10 μM histamine on the EPSPs, indicating that this effect is not mediated by H_1 and H_2 receptor subtypes. In contrast, as shown in figure 2B, the suppressive effect of 10 μM histamine on EPSPs was never observed in the presence of 20μM thioperamide, a selective H_3 receptor antagonist. Furthermore, 2 μM R-alpha-methylhistamine, an H_3 receptor agonist, could mimic the suppressive effects of histamine on EPSPs (70.0±3.4% of the baseline) ($P<0.01$, n=13, figure 2C). These results suggest that the histamine-induced suppression of glutamatergic synaptic transmission is mediated by the H_3 receptor subtype.

The suppression of EPSPs by histamine was accompanied by an increase in paired-pulse facilitation (PPF). Application of 10 μM histamine for 15 min increased PPF of the slope of EPSPs from 1.14±0.04 in the control to 1.34±0.14 (n=5, $P<0.05$, Fig3C), suggesting a presynaptic locus of histamine action. To obtain further evidence on whether the suppressive action of histamine is mediated presynaptically, we examined whether

histamine depressed the AMPA/kainate receptor-mediated component of EPSPs (AMPA/kainate-EPSPs) and the NMDA receptor-mediated component (NMDA-EPSPs) to an equivalent extent. If both AMPA/kainate and NMDA receptor-mediated responses are suppressed to a similar extent, then it is more likely that histamine acts presynaptically suppressing the release of glutamate, rather than postsynaptically affecting selectively the AMPA/kainate receptors. In the presence of D-APV in the perfusion solution to block the NMDA-EPSPs, 10 μM histamine depressed the AMPA/kainate-EPSPs to 70.2±4.9% of baseline (n=11), which was not significantly different from the histamine-induced suppression of EPSPs in the absence of D-APV. Then, we examined the effect of histamine on the NMDA-EPSPs. In the presence of 10 μM biccuculline, 5μM SCH50911 and 20μM LY293558 in the perfusion solution and with an increased stimulus intensity (Rainnie et al., 1991;Chen et al., 2003), the EC-evoked NMDA-EPSPs can be recorded in BLA neurons. As reported in our previous studies (Chen et al., 2003), this slow component is small at resting membrane potential, and can be completely blocked by application of the NMDA receptor antagonist APV (50μM) , indicating that this component is mediated by NMDA receptors. As shown in figure 3A, bath application of 10 μM histamine for 15 min depressed the peak amplitude of NMDA-EPSPs (69.5 ± 2.3% of baseline, n=12) to a similar extent as observed in the AMPA/kainate-EPSPs ($P>0.05$). The suppressive effect of 10 μM histamine on NMDA-EPSPs could also be prevented in the presence of 20 μM thioperamide, a selective H_3 receptor antagonist (n=6, figure 3B). In addition, 10 μM histamine increased PPF of the slope of NMDA-EPSPs from 1.29±0.07 in the control to 1.42±0.08 ($P<0.05$, n=5). These

data suggest that the histamine-induced suppression of NMDA-EPSPs is associated with a presynaptically located H_3 receptor.

3. The pharmacological characteristics of the histamine-evoked potentiation of EPSPs.

Previous studies have shown that NMDA receptors may contribute to the histamine receptor-mediated long-term potentiation of synaptic transmission (Kostopoulos et al., 1988;Vorobjev et al., 1993). Therefore, we examined whether blockade of NMDA receptors might prevent the development of histamine-induced potentiation of EPSPs in the amygdala circuitry. In the presence of 50 μM D-APV, a selective NMDA receptor antagonist, 20 μM histamine enhanced the amplitude of EPSPs to 336±79% of control levels (n=6; $P<0.01$) in six BLA neurons recorded, which was not significantly different from that without D-APV pre-application, indicating that NMDA receptors are not involved in the histamine-induced potentiation of EPSPs.

The histamine-induced potentiation of glutamatergic transmission appears to be specific to the AMPA/kainate component of the EPSPs. In all neurons examined (n=18), NMDA receptor-mediated EPSPs were consistently reduced by histamine. Even when the H_3 receptors (which mediate the suppression) were blocked, no potentiation of the NMDA-EPSPs was observed (figure 3A and 3B).

The differential modulation of AMPA/Kainate-EPSPs versus NMDA-EPSPs by histamine suggests a postsynaptic locus of potentiating actions. Histamine H_1 and H_2 receptors are postsynaptically located. Thus, the histamine-induced potentiation of EPSPs could be mediated by the H_1 or H_2 receptor subtype. However, the H_1 receptor antagonist mepyramine (up to 50 μM, n=11) or the H_2 receptor antagonist cimetidine (up to 100 μM,

n=7), did not significantly inhibit the histamine-induced potentiation of EPSPs (figure 4A, 4C and 4D). Furthermore, in the presence of the very potent H_1 receptor antagonist triprolidine (20 μM; n=2) and the H_2 receptor antagonist tiotidine (5 μM; n=2), the histamine-induced potentiation of EPSPs was still unaffected. Similarly, the high concentration of the H_3 receptor antagonist, thioperamide, did not affect the histamine-induced potentiation (Fig. 4B, 4D). However, thioperamide eliminated the modest, transient depression that often proceeded the potentiation (Fig. 4B).

As described above, histamine, at the low concentration of 200 nM, could not produce a significant suppression of the EPSPs recorded in 12 out of 17 neurons (Fig. 2). However, histamine at this concentration (200 nM) was still capable of inducing a robust potentiation of EPSPs in five out of 17 BLA neurons (311±40% of baseline; $P<0.01$, Figure 5). This particularly high potency of histamine in inducing potentiation raises the possibility that the potentiating action may be mediated by a particularly high affinity isoform of the H_3 receptor distinct from the classical H_3 receptor isoform. To investigate this possibility, clobenpropit, an H_3 receptor antagonist with 10 fold more potency in antagonizing the H_3 receptor than thioperamide (Schlicker et al., 1994) was tested. In the presence of 10μM clobenpropit, the potentiation effect of histamine on EPSPs still remained unaffected (n=3).

4. Bidirectional effects of histamine on BLA field potentials.

Since histamine produces opposite effects on EPSPs recorded from the different BLA neurons, the net effect of histamine on excitability of the BLA nucleus needed to be further determined. In the next series of experiments, field potential recordings were used to examine the effects of histamine on population neuronal responses in the BLA.

Field potentials evoked in the BLA by stimulation of the external capsule, as shown in our previous studies (Aroniadou-Anderjaska et al., 2001;Chen et al., 2003), consist of one major, negative component that corresponds in time course to the EPSPs recorded intracellularly from BLA pyramidal cells, and is mediated by AMPA/kainate receptors (Aroniadou-Anderjaska et al., 2001). Bath application of 20 μM histamine for 15 min reduced field potentials (70.6±18.7% of the control peak amplitude, n=15, $p<0.05$), However, at 200 nM, histamine induced potentiation of the peak amplitude of field potentials (138.8 ± 18.2% of control, n=12, $P<0.05$**),** in all slices examined (Figure 6A). Moreover, in the presence of the H_3 receptor antagonist thioperamide (20 μM), the effect of 20 μM histamine consistently reversed to producing potentiation of field potentials (141.2±13.6% of baseline; n=12; $P<0.01$) in all amygdala slices examined (Figure 6D). The H_3 antagonists had no effect on the potentiation of the field potentials produced by 200 nM histamine; thus, in the presence of clobenpropit (10 μM), 200 nM histamine increased the field potential amplitude to 143.0 ± 18.9 % of the control (n=6; $P>0.05$, Figure 6B). Furthermore, as shown in the figure 6C, the potentiation induced by 200 nM histamine remained unaffected (139.9± 12.5% of the control amplitude, n=6) in the presence of all three histamine receptor antagonists, mepyramine (5μM, H_1 receptor antagonist), thioperamide (10 μM, H_3 receptor antagonist) and cimetidine (10μM, H_2 receptor antagonist). These results further support the contention that the depressive effect of histamine on excitatory synaptic transmission in the BLA is mediated by H_3 receptors, while the potentiating effect is mediated via a mechanism different from the classical H_1, H_2 and H_3 receptors.

The histamine-induced potentiation was not accompanied by the significant change in PPF of the slope of field potentials (1.12±0.08 versus 1.13 ±0.06), further supporting a postsynaptic locus of potentiating action of histamine. In the presence of the selective H_3 receptor antagonist thioperamide, the EC50 for the potentiating action of histamine is 49 nM, much lower than the EC50 (470 nM) of H_3 receptor-mediated suppression of EPSPs in the BLA (Figure 7).

DISCUSSION

The main finding of this study is that histamine can suppress or potentiate glutamatergic synaptic transmission in different BLA neurons through different mechanisms. The histamine-induced suppression of glutamatergic synaptic transmission is presynaptically mediated by the H_3 receptor, while the potentiating effect of histamine appears to be postsynaptically mediated, unrelated to currently known histamine receptors.

H_3 receptor-mediated suppression of synaptic transmission in the BLA

At the concentration of 20 μM, histamine suppressed EPSPs in about two third of the BLA neurons recorded intracellularly. Field potentials were consistently suppressed by this concentration of histamine, as long as H_3 receptors were unblocked. Several observations in the current study support the notion that this inhibitory effect of histamine is mediated by presynaptic H_3 receptors located on the terminals of the EC fibers. First, the histamine-induced suppression of synaptic transmission was completely blocked by the selective H_3 receptor antagonist thioperamide, and was mimicked by the selective H_3 receptor agonist R-alpha-methylhistamine. Second, the histamine-induced suppression of the EPSPs was associated with an increase in PPF of the slope of the EPSPs. PPF is considered to be a presynaptic phenomenon, resulting from a transient increase of presynaptic Ca^{2+} and has become a standard means of gauging the presynaptic action of neuromodulators (Zucker, 1989;Zucker and Regehr, 2002). In previous studies, the PPF protocol has been used to demonstrate a presynaptic mechanism in both excitatory and inhibitory terminals in the BLA (Zinebi et al., 2001;Braga et al., 2002). Third, 20 μM

histamine attenuated the NMDA-EPSPs to the same extent as AMPA/kainate-EPSPs, and the suppressive effects on both NMDA and AMPA/kainate-EPSPs were abolished by the presence of the H_3 antagonist thioperamide.

The EC_{50} value for the suppressive action of histamine in BLA was 470 nM, consistent with histamine potency for the H_3 heteroreceptor identified in glutamatergic terminals of the dentate gyrus (Brown and Reymann, 1996). However, in corticostriatal glutamatergic terminals, the EC50 value for the H_3 receptor is 1.6 μM, much higher than that reported here and in the dentate gyrus (Doreulee et al., 2001). The inhibition of glutamatergic synaptic transmission described here also showed some significant differences from the H_3-mediated inhibition of the release of other transmitters (Schwartz et al., 1991b). A higher EC_{50} for H_3 receptor-mediated glutamatergic synaptic transmission in our study and other similar studies, compared to nanomolar potency for histaminergic inhibition of histamine release may be due to the brain slice preparation and the perfusion system we used. Bath application of agonist and antagonists to the brain slices in interface chambers, is unlike the typical binding study in which drugs are incubated with a membrane homogenate. The concentration of drugs at the synapses of the slices is likely to be somewhat lower than that in the perfusion solution. Nevertheless, the differences in the EC50 value for the H_3 receptor in different brain regions more likely reflect the existence of diversified isoforms of the H_3 receptor with different pharmacological profiles. In fact, multiple isoforms of the H_3 receptor have recently been cloned in the human, rat and guinea pig brain (Tardivel-Lacombe et al., 2000;Drutel et al., 2001;Wellendorph et al., 2002). Thus far, at lease six isoforms of the H_3 receptor have been identified in the human brain and these isoforms reveal up to 10-15 fold differences

in their affinities to the different H_3 histamine agonists. In rat brain, at least three functional isoforms of the H_3 receptor have been currently identified. Although the pharmacological profiles of these three isoforms in rat brain are quite similar (Drutel et al., 2001), the possibility of the existence of additional as yet undiscovered H_3 receptor isoforms in rat brain with distinct pharmacological profiles can not be ruled out. Indeed, West et al. identified two distinct histamine H_3 receptor binding sites in rat brain tissue using a radioligand (Nα-[^{3}H] methylhistamine) binding assay (West, Jr. et al., 1990). Their experiments with inhibition of Nα-[^{3}H] methylhistamine binding by thioperamide and burimamide, two H_3 receptor antagonists, discriminate two sites with 12 to 350 fold differences in their affinities in responding to these antagonists respectively, suggesting two H_3 receptor isoforms with distinct pharmacological profiles (West, Jr. et al., 1990). The cloning of these isoforms from rat brain is to be expected in the future.

Potentiating effect of histamine on EPSPs in the BLA

A robust potentiation (more than three times) of EPSPs by histamine was observed in about one third of the recorded neurons when different concentrations of histamine (from 0.2 μM to 20 μM) were applied to the amygdala slices. The potentiating action of histamine has also been consistently observed in field potential recordings when the concentration of histamine is low (200 nM). 20 μM histamine induced potentiation only when H_3 receptors were blocked. Since EPSPs recorded from only one small portion of BLA neurons could be potentiated by histamine, and the recorded field potentials reflect the population EPSPs of all BLA neurons, the potentiating effect of histamine on the field potentials, as expected, is much smaller than the potentiating effect of histamine on individual neurons.

That BLA neurons differ in response to histamine reflects heterogeneity of BLA neurons. BLA neurons have been previously categorized, according to their electrophysiological and morphological features, into three major cell types: pyramidal cells, late-firing neurons and fast-firing neurons (Braga et al., 2004;Rainnie et al., 1993;Washburn and Moises, 1992). The electrophysiological properties of all BLA neurons included in the current study were typical of those observed in pyramidal neurons; no differences were evident between the neurons displaying potentiation of EPSPs versus the neurons displaying suppression of EPSPs in response to histamine. Whether these two groups of neurons have distinct morphological characteristics remains to be determined.

The histamine-potentiated EPSPs could be completely blocked by a combination of AMPA/kainate antagonists LY293558 and NMDA receptor antagonist D-APV, indicating that histamine-induced potentiation is not due to an additional component recruited by histamine. The potentiating effect of histamine appears to be due to a postsynaptic modulation of AMPA/kainate receptors by histamine. Firstly, this potentiating effect is not accompanied by the change in the PPF. Secondly, in our experiments, NMDA-EPSPs were consistently suppressed by histamine, without observing the potentiating action of histamine on NMDA-EPSPs. In addition, the histamine-induced potentiation of EPSPs was unaffected by the presence of the NMDA receptor antagonist D-APV. These results suggest that the histamine-induced potentiation of glutamatergic synaptic transmission in the BLA is specifically expressed in the AMPA/kainate component of the EPSPs, and does not require activation of NMDA receptors.

Facilitatory effects of histamine on neuronal excitability and synaptic function were previously demonstrated in the hippocampus to be mediated by H_1 or H_2 receptor activation (Brown et al., 1995;Barbara et al., 2002;Korotkova et al., 2002). However, our results demonstrate that neither H_1 nor H_2 receptor subtypes mediate the histamine-induced potentiation of EPSPs in the BLA, as the potentiating effect of histamine was not blocked by the H_1 receptor antagonist mepyramine (up to 50 μM) or the H_2 receptor antagonists cimetidine (up to 100 μM). Mepyramine, at 5 μM, and cimetidine, at 50 μM have been demonstrated to fully antagonize the H_1 receptor and H_2 receptor-mediated effects, respectively, induced by up to 30 μM histamine, in *in vitro* acute brain slice preparation (Brown et al., 1995;Barbara et al., 2002;Korotkova et al., 2002). Thus, it is unlikely that failure of cimetidine and mepyramine to block the histaminergic potentiation is due to their insufficient concentrations. In addition, the more potent H_1 receptor antagonist triprolidine (20 μM) and the H_2 receptor antagonist tiotidine (5 μM) also failed to antagonize the histamine action. These results are not surprising considering that there is no significant H_1 and H_2 receptor expression in the rat basolateral amygdala (Ryu et al., 1995b;Honrubia et al., 2000;Karlstedt et al., 2001). Our results also indicate that the potentiating action of histamine is unlikely to be mediated by the classical H_3 receptor, at least not by the same H_3 receptor isoform as that mediating the suppressive action of histamine. This action has a much lower EC_{50} (49 nM) than the H_3 receptor-mediated suppressive effect characterized here. As mentioned previously, the brain expresses multiple functional H_3 receptor isoforms, some of which could have much lower affinity than other H_3 receptor isoforms. This raises the possibility that the histamine-induced potentiation is associated with a novel high-affinity H_3 receptor

isoform different from the classical H_3 receptor. Nevertheless, in the presence of high concentration of the H_3 receptor antagonists thioperamide and clobenpropit, histamine was still able to potentiate EPSPs, although the suppressive effect on EPSPs was prevented by these antagonists. Whether or not there are the high-affinity H_3 isoforms that are not blocked by the classical H_3 antagonists, and might mediate the synaptic potentiation that we observed remains to be determined.

Recently, a novel histamine receptor, the H_4 receptor, has been cloned and characterized by many laboratories simultaneously (Oda et al., 2000;Liu et al., 2001;Morse et al., 2001). The pharmacological profile of H_4 receptor is quite similar to the H_3 receptor (Oda et al., 2000;Liu et al., 2001). The expression of this receptor is primarily restricted to the bone marrow and eosinophils (Oda et al., 2000;Liu et al., 2001;Morse et al., 2001). In addition, the selective H_3 receptor antagonist thioperamide, is also a potent antagonist for this receptor (Raible et al., 1992;Morse et al., 2001). Therefore, it is unlikely that the histamine–evoked potentiation of EPSPs in the BLA is mediated by the H_4 receptor.

Functional roles of histamine in the amygdala

In summary, histamine, depending on the concentrations, decreases or increases excitatory synaptic transmission in the amygdale through the presynaptic H_3 receptor and a currently unknown mechanism respectively. In the *in vivo* condition, low concentrations of extracellular histamine released from histaminergic terminals during low activity of tuberomammillary (TM) neurons can be expected to facilitate the excitatory synaptic transmission in the BLA. Considering the central role of the BLA in fear conditioning and the consolidation of emotional memories (Rogan and LeDoux,

1996;Nader and LeDoux, 1999;Aggleton JP and Sauder RC, 2000), such facilitation of excitatory synaptic transmission may serve to alert emotional consciousness or to enhance the level of arousal. However, the extracellular concentrations of histamine released during intensive activities of TM neurons can reach and activate presynaptic H_3 receptors, thus preventing or dampening excitation of the amygdala during external or internal over-stimulation to maintain mood stability, or to prevent panic attack and social phobias (Schneider et al., 1999;Layton and Krikorian, 2002;Layton and Krikorian, 2002;Liberzon et al., 2003). In this respect, the histaminergic system provides an important mechanism for refining activation of the amygdala, and may play an important role in the modulation of amygdala-related biological processes, such as fear, epilepsy and affective disorders.

Acknowledgement.

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Fig. 1. Effects of 20 μM histamine on EPSPs, resting membrane potential and input resistance of neurons in the BLA. (A1, A2, B1, B2). A0 shows the recording and stimulating site in the amygdala slices. Histamine, at a concentration of 20 μM, produced opposite effects on intracellularly recorded EPSPs, evoked by stimulation of the EC. A1 and A2 show examples of neurons where histamine reduced the peak amplitude of EPSP, and B1 and B2 show recording from neurons where histamine increased the peak amplitude of EPSP. A1 and B1 show the continuous intracellular chart recordings of the neurons, while A2 and B2 show that the two traces before and during histamine application are superimposed. To examine the electrophysiological properties of the neurons, evoked EPSPs are followed by a depolarizing current pulse passing through the recording electrode to produce action potentials. Note that histamine had no effect on resting membrane potential, firing pattern and input resistance of BLA neurons, while producing marked effects on the peak amplitude of EPSPs. The inset shows the blockade of an EPSP by the kainate/AMPA receptor antagonist Ly293558. A3 and B3 represent the cumulative data (mean±S.E.M) of the peak amplitude of EPSPs from two groups of neurons in response to 20 μM histamine. A3 shows data from 20 neurons where 20 μM histamine reduced the amplitude of the EPSPs. B3 shows data from 16 neurons where 20 μM histamine increased the amplitude of the EPSPs. For each neuron included in the calculation of the mean, the average of six consecutive EPSPs, evoked at 0.1 Hz, was expressed as a percentage of the mean of 60 responses before application of 20 μM histamine. The sample EPSP traces were the average of six consecutive EPSP traces and were taken at the times indicated on the plot by the letters a, b, c and d. The duration of drug application is indicated by a horizontal bar.

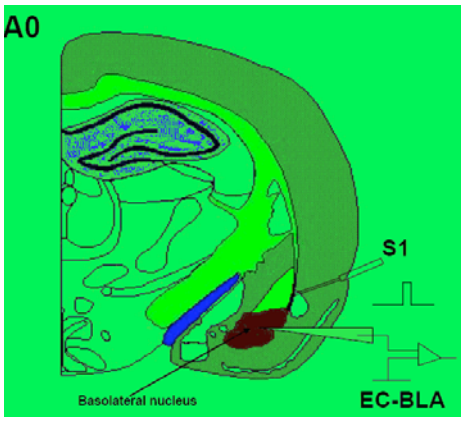

A0
S1
Basolateral nucleus
EC-BLA

Figure 1

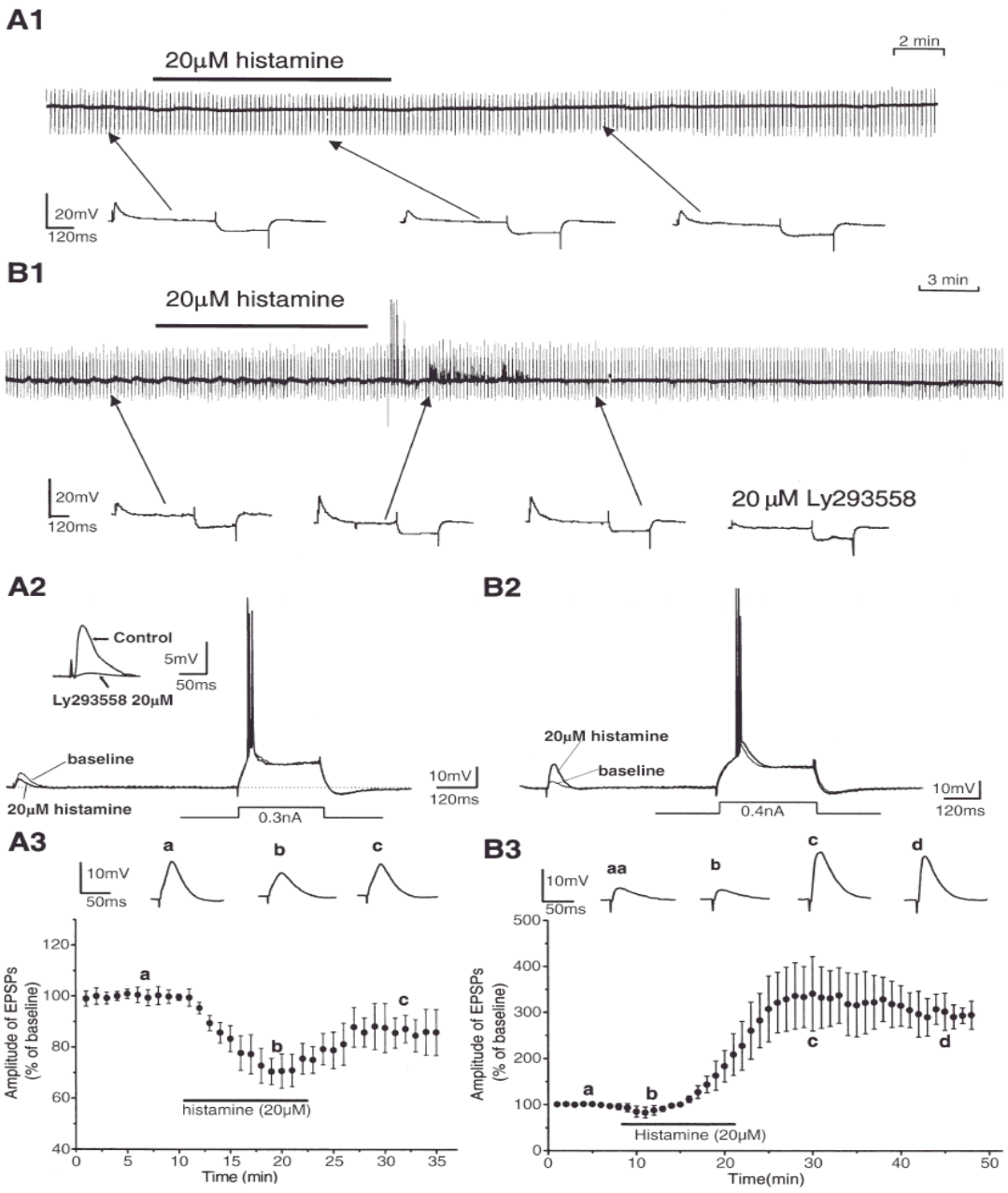

A1
20μM histamine
2 min
20mV
120ms
B1
20μM histamine
3 min
20mV
120ms
20 μM Ly293558
A2
Control
5mV
50ms
Ly293558 20μM
baseline
20μM histamine
10mV
120ms
0.3nA
B2
20μM histamine
baseline
10mV
120ms
0.4nA
A3
a
b
c
10mV
50ms
Amplitude of EPSPs (% of baseline)
histamine (20μM)
Time (min)
B3
aa
b
c
d
10mV
50ms
Amplitude of EPSPs (% of baseline)
Histamine (20μM)
Time(min)

Table 1.
Effect of 20μM histamine on the passive and active neuronal properties [a].

BLA neurons		Control	20M histamine
Group I	Resting potential	68.6±1.9(n=7)	67.2±2.3
	Input Resistance	50.7±10.5 MΩ (n=6)	52.1±12.1 MΩ
	Number of action potential	5.8±0.7(n=7)	5.9±1.1
Group II	Resting potential	69.7±2.6 (n=7)	68.9±2.8
	Input Resistance	49.2±7.4MΩ(n=6)	51.1±8.8M Ω
	Number of action potential	5.7±1.2 (n=6)	5.8±1.5

[a]Application of 20 μM histamine for 15 min didn't significantly change resting membrane potential, input resistance measured during 250ms, -0.1nA hyperpolarizing current pulse, or the number of action potential elicited by a 250ms,0.6 nA depolarizing current pulse in both groups of BLA neurons (Group I neurons: the neurons with only depression response of EPSPs to histamine; Group II neurons: the neurons with the potentiation response of EPSPs to histamine). Note the passive and active neuronal properties in both groups of neurons are not significantly different.

Figure 2

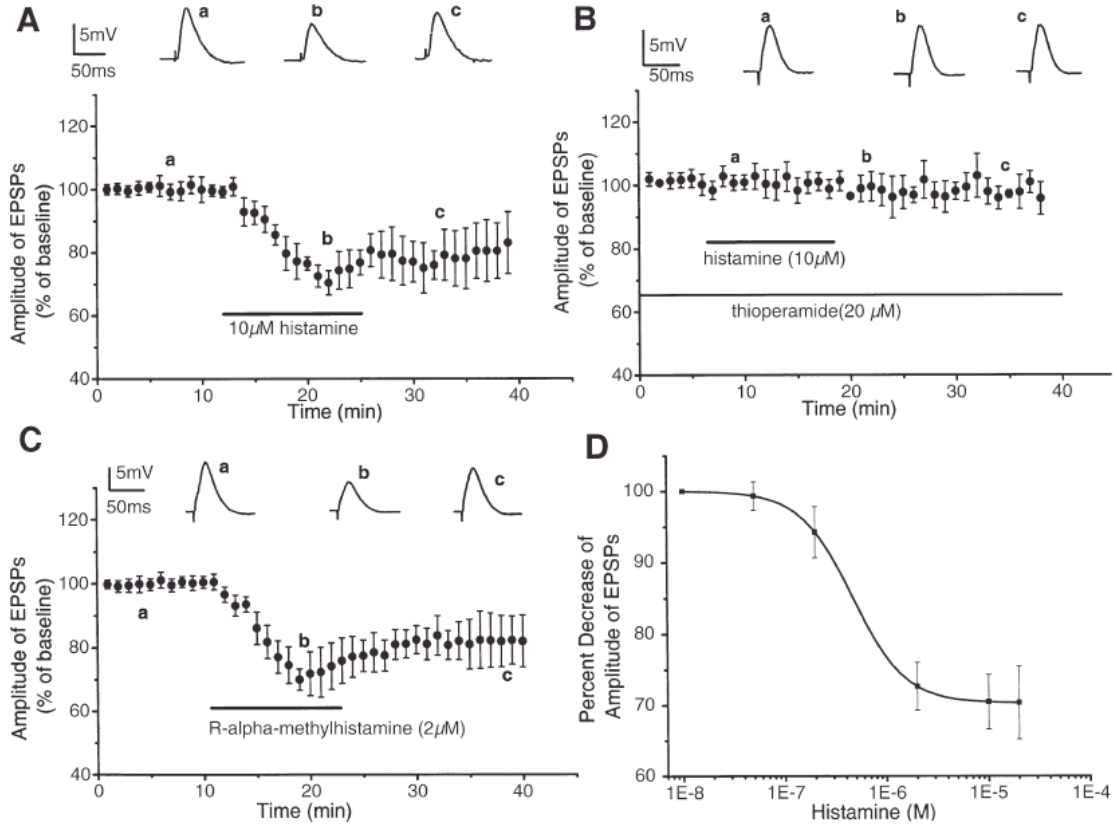


Fig. 2. The histamine-induced suppression is mediated by histamine H_3 receptors. (A) Cumulative data (mean ± S.E.M) from seven neurons, showing a decrease in the EPSP amplitude by bath application of 10 μM histamine. Sample traces, at the top, are the average of six consecutive EPSP traces and were recorded at the points of time indicated by the letters a, b, and c. (B) In the presence of the H_3 receptor antagonist thioperamide, histamine-induced suppression was abolished. These cumulative data are from six neurons. (C) The H_3 receptor agonist *R*-α-methylhistamine mimicked the effects of 10 μM histamine-induced suppression of EPSP. These cumulative data are from 13 neurons. (D) The dose-response relationship for the suppression of EPSP amplitude by histamine. The percentage decrease of EPSP amplitude is plotted against the concentrations of histamine on a logarithmic scale (0.05 μM, *n*=2; 0.2 μM, *n*=6; 2 μM, *n*=5; 10 μM, *n*=7). The points were fit by a sigmoidal dose-response equation with an estimated EC_{50} of 0.47 μM.

Figure 3

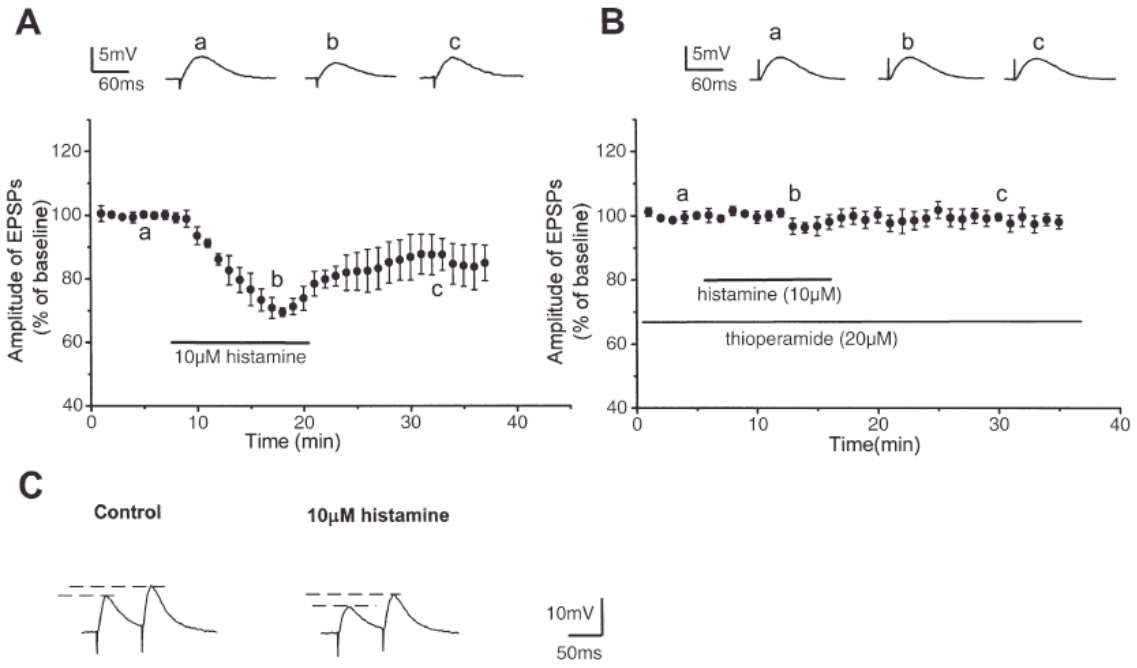


Fig. 3. The suppression of EPSPs by histamine involves a presynaptic mechanism. (A) Similar to the effects of histamine on AMPA/kainate receptor-mediated EPSPs, isolated NMDA receptor-mediated EPSPs were also reduced by histamine. The plot shows group data (mean±S.E.M) of the peak amplitude of NMDA receptor-mediated EPSPs from seven neurons. Example traces are shown in the top. These EPSPs were recorded at the points of time indicated on the plot by the letters a, b and c. (B) In the presence of the H_3 receptor antagonist thioperamide (20 μM), histamine had no effect on the NMDA receptor-mediated EPSPs. The cumulative data are from six neurons. (C) An example of EPSPs evoked by paired-pulse stimulation of the EC, at an inter-stimulus interval of 70 ms. In addition to reducing the amplitude of the first EPSP, histamine also increased PPF.

Figure 4

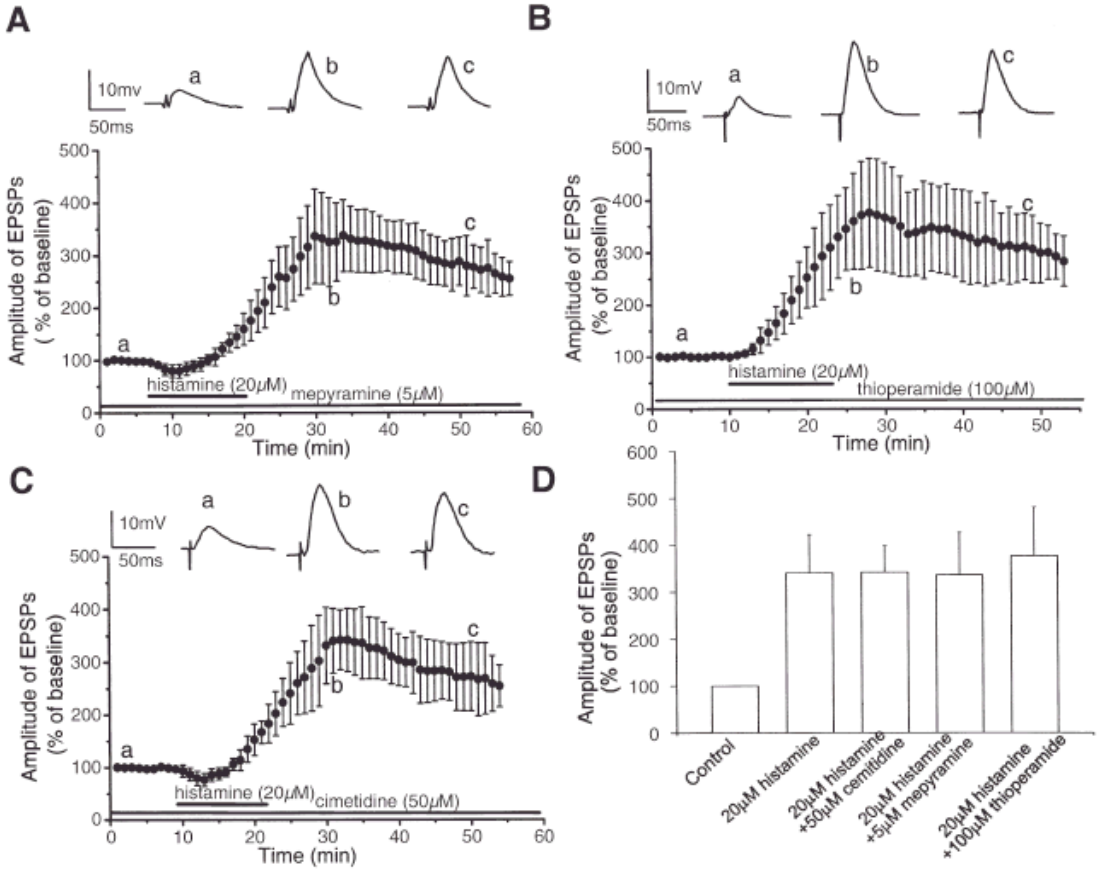


Fig. 4. Histamine-induced potentiation of EPSPs could not be blocked by H_1, H_2 or H_3 antagonists. A, B, and C represent cumulative data (mean ± S.E.M) of peak amplitudes of EPSPs recorded intracellularly from BLA neurons that displayed a potentiating response to application of 20 μM histamine. In the presence of 5 μM mepyramine (A, n=6), 100 μM thioperamide (B, n=6), or 50 μM cimetidine (C, n=4) in the perfusion solution, histamine-induced potentiation of EPSPs remained unaffected. The sample EPSP traces were the average of six consecutive EPSP traces and taken at the times indicated on the plots by the letters a, b and c. Bars denote the delivery periods of drugs. (D) Bar graph summarizes the potentiating effects of histamine (maximal peak amplitude of EPSPs) when applied alone, or in the presence of 5 μM mepyramine, 100 μM thioperamide, or 50 μM cimetidine. There was no significant difference in the maximal potentiation of EPSPs induced by histamine alone, or by histamine in the presence of any of the three histamine receptor antagonists.

Figure 5

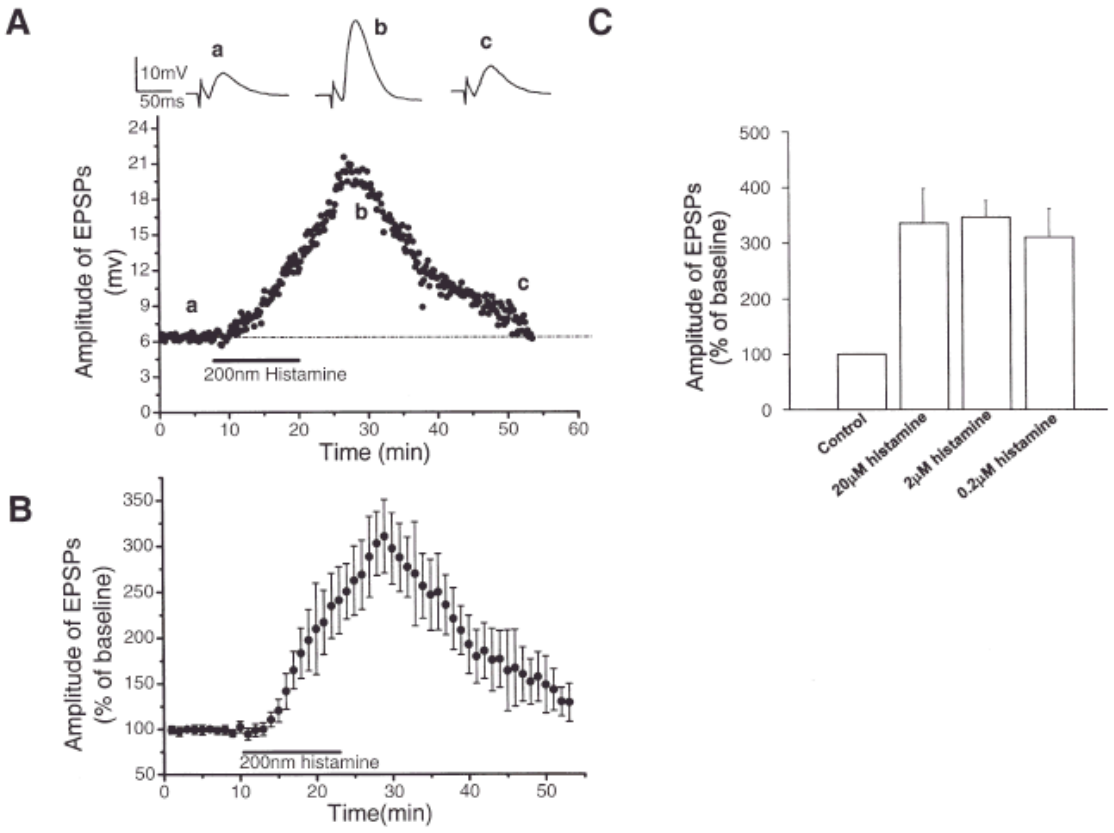


Fig. 5. Histamine, at a low concentration, potentiated the peak amplitude of EPSPs. (A, B). A shows an example of neurons where 200 nM histamine potentiated EPSPs, while B represents cumulative data (mean ±S.E.M) from five neurons where 200 nM histamine potentiated EPSPs. The sample EPSP traces shown in the top were recorded at the times indicated on the plot by the letters a, b and c. (C) Maximal potentiation of EPSPs induced by 20 μM histamine (*n*=16), 2 μM histamine (*n*=2), or 0.2 μM histamine (*n*=5) is shown. There was no significant difference in the maximal potentiation induced by these three concentrations of histamine.

Figure 6

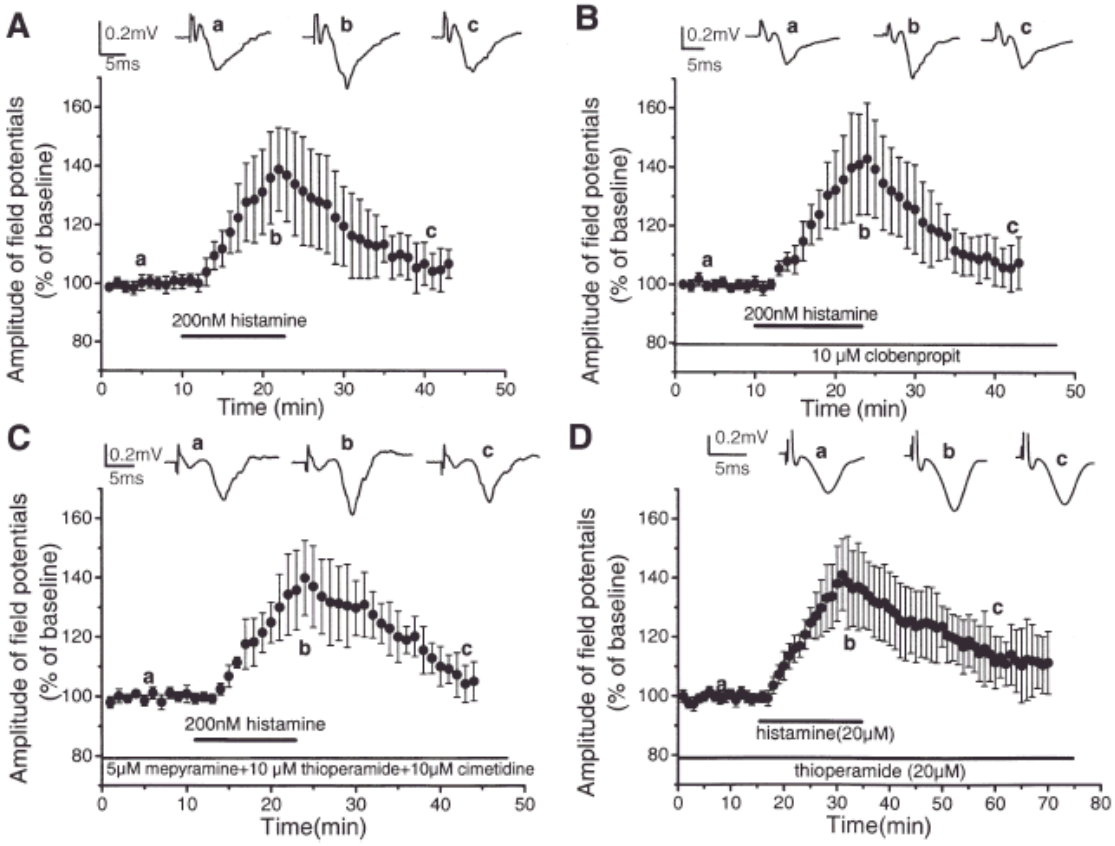


Fig. 6. The potentiating effect of histamine on field potentials (fEPSPs) was not affected in the presence of H_1, H_2, or H_3 receptor antagonists. (A) Histamine (200 nM) was shown to consistently potentiate the peak amplitude of fEPSPs. The group data (mean ±S.E.M), *n*=12) of the peak amplitude (% of baseline) of the EPSPs are shown. The histamine-induced potentiation of the fEPSPs was not blocked by the H_3 receptor antagonist clobenpropit (B, *n*=6) or by the presence of all three histamine receptor antagonists combined, mepyramine (5 μM), thioperamide (10 μM) and cimetidine (10 μM) in the perfusion solution (C, *n*=6). (D) In the presence of thioperamide in the perfusion solution, the high concentration of histamine (20 μM) also consistently potentiated fEPSPs (*n*=12). Traces of fEPSPs above each plot are the average of six consecutive responses to stimulation at 0.1 Hz, and were recorded at the points of time indicated on the plots by the letters a, b, and c.

Figure 7

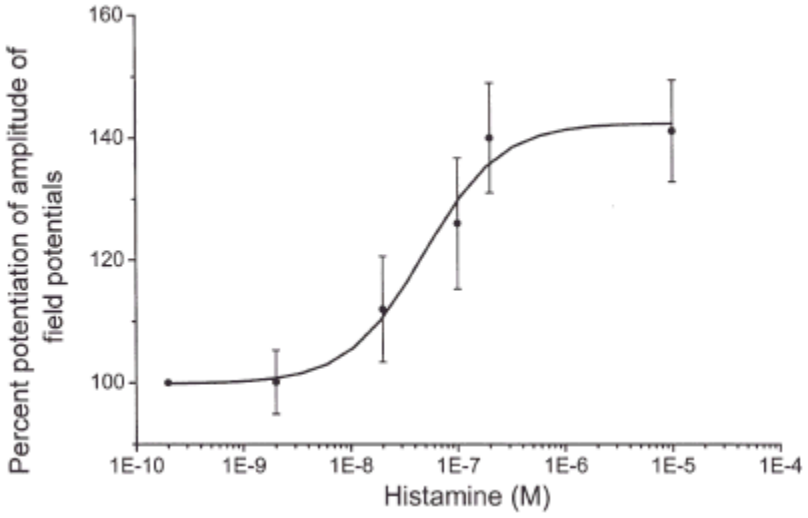


Fig. 7. Dose-response relationship for the potentiation of field potential amplitude by histamine. The percentage increase of EPSP amplitude by histamine in presence of 1 μM thioperamide is plotted against the concentrations of histamine on a logarithmic scale. (2 nM, *n*=9; 20 nM, *n*=10; 100 nM, *n*=9; 200 nM, *n*=7; 10 μM, *n*=7). The points were fit by a sigmoidal dose-response equation with an estimated EC_{50} of 49.0 nM

CHAPTER 5

Effect of Traumatic Stress on Histamine H_3 Receptor and α_2 Adrenoceptor-Mediated Suppression of fEPSPs in BLA *in vitro*

Xiaolong Jiang, He Li

ABSTRACT

The histaminergic system and noradrenergic system in the brain, with dense innervations of the amygdala, prefrontal cortex, and hypothalamus, play crucial roles in stress/danger responses. Their dysregulation may participate in the pathogenesis of multiple anxiety and stress disorders. In order to examine the possible involvement of these two systems in these disorders, the present study investigated whether these two aminergic systems in the rat amygdala, a central brain region participating in stress response, could be altered by inescapable stress, a protocol which could stably induce the physiological and behavioral abnormality associated with anxiety and stress disorders. In control animals, histamine suppressed excitability of the basolateral amygdala and this effect was mediated by the H_3 histamine receptor. The histamine H_3 receptor agonist, R-α-methylhistamine (2 μM), decreased fEPSPs recorded in the basolateral amygdala (BLA) to 66.2±9.3% of the baseline values. In stressed animals, R-α-methylhistamine (2μM) decreased the fEPSPs to 62.4 ±7.5% of the baseline values, which is not significantly different from the level of suppression seen in non-stressed animals. In the BLA, norepinephrine suppressed excitability and this effect was partially mediated by the α_2 adrenoceptor. In control animals, the selective α_2 adrenoceptor agonist, clonidine (50 μM), suppressed fEPSPs recorded in the BLA to 61.2 ±5.7% of the baseline value. In stressed animals sacrificed immediately after stress, clonidine (50 μM) suppressed the fEPSPs to 55.6±6.2% of the baseline values. In stressed animals sacrificed 7 days after stress, clonidine (50 μM) suppressed fEPSPs to 60.7 ± 13.9 % of the baseline values, which was not significantly different from the levels seen in controls (57.2 ± 7.3 % of the baseline values). The results indicate that the α_2 adrenoceptor and histamine H_3 receptor

are not altered by traumatic stress. It can't be excluded that these receptors in other brain regions or other monoaminergic receptor subtypes may be involved in the pathophysiology of stress.

INTRODUCTION

As the previous study has shown, traumatic stress could impair 5-HT_{2A} receptor signaling in the BLA. Whether or not this stress-induced alteration is specific to the serotonergic system is not known. Therefore, we examined alterations of the noradrenergic system and histamine system in the amygdala as a function of stress considering that dysregulation of these two systems has also been implicated in anxiety and stress disorders.

The norepinephrine-releasing neurons are primarily located in the locus coeruleus (LC). It provides the majority of norepinephrine for the forebrain, including the amygdala, prefrontal cortex and hippocampus, to modulate the stress/danger response, learning, and memory (Berridge and Waterhouse, 2003). Stressful experiences dramatically increase norepinephrine in the amygdala (Quirarte et al., 1998;Galvez et al., 1996), and increased noradrenergic neurotransmission in the amygdala during stress is involved in enhanced encoding of emotional memories, sensitization, and fear conditioning (McGaugh and Roozendaal, 2002;Southwick et al., 1999a;McGaugh et al., 1990). Nevertheless, the effect of norepinephrine on amygdala's excitability appears to be inhibitory. This inhibitory effect is mediated by the α_1 adrenoceptor and α_2 adrenoceptors (Braga et al., 2004;DeBock et al., 2003).

The noradrenergic system in the brain could be affected by stress, especially by chronic and traumatic stress. Such stress could downregulate the α_2 adrenoceptor in the locus coeruleus (Simson and Weiss, 1988;Flugge, 1996) and prefrontal cortex (Flugge, 1996). Chronic stress in rats has also been shown to block the reduction of locomotor activity normally associated with the administration of the selective α_2 adrenoceptor

agonist, clonidine, suggesting a decreased responsiveness of the α_2 adrenoceptor following chronic stress (Cancela et al., 1988). Animals raised under the severe conditions of early deprivation, a severe stressor for the infant, manifested blunted behavioral response to the α_2 adrenoceptor antagonist, yohimbine, suggesting that early stress altered α_2 adrenoceptor function in these animals (Coplan et al., 1992). In addition, a decrease in the density of the α_2 adrenoceptor specific to the hippocampus and amygdala has been associated with acute cold-restraint stress in rats (Nukina et al., 1987). Our laboratory has demonstrated that traumatic stress severely impaired α_1 adrenoceptor-mediated facilitation of GABA release in the BLA (Braga et al., 2004). However, whether the α_2 adrenoceptor could also experience a similar change in the amygdala after exposure to stress has not been examined.

The rat histaminergic system, like the serotonergic and noradrenergic system, also highly innervates the brain regions that participate in stress/emotion responses, including the hypothalamus, basal forebrain and amygdala (Panula et al., 1989). Through its innervations of these brain regions, this system appears to play an important role in modulating the stress/emotion response (Brown et al., 2001). As shown in Chapter 4, histamine suppressed amygdala excitability through a presynaptic histamine H_3 receptor. Although the change of this system and the H_3 receptor has not been extensively reported in stress-related disorders, it is worthy to investigate the effect of chronic, traumatic stress on this system, not only because this system may serve as a negative control of the whole study, but also it may provide a possible new research direction for psychiatry disorders.

Thus, the present study investigated whether α_2 adrenoceptor and H_3 receptor-mediated electrophysiological function in the amygdala could be altered by traumatic

stress. The results demonstrated that stress could not significantly change the signaling of these two receptors in the amygdala.

MATERIALS AND METHODS

Amygdala slice preparation.

Male Sprague-Dawley rats weighing 75–150 g (4-6 weeks) were decapitated after light anesthesia with halothane, the brains were rapidly removed, and 450-µm-thick transverse slices of the amygdala were cut from tissue blocks with a Vibratome (Technical Products International, St. Louis, Missouri). The slices were preincubated in artificial cerebrospinal fluid (ACSF), continuously bubbled at room temperature (23°C) with 95% O_2 /5% CO_2 to maintain a pH of 7.4, for at least 1 hour before use. The ACSF contained 117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$ and 11 mM glucose.

Field potential recording.

Slices were transferred to an interface chamber that was continually superfused with ACSF at a rate of 1–2 ml/min. The temperature of perfusion solution in the chamber was maintained at 32°C through a TC-202A Bipolar temperature controller (Harvard Apparatus, Inc., Holliston, Ma, USA). Microelectrodes were pulled from microfiber-filled borosilicate capillaries (OD 1.0 mm, ID 0.78 mm) using a Flaming-Brown horizontal micropipette puller (Sutter Instruments, Novato, CA, USA). The resistance of electrodes filled with 3 M NaCl ranged from 2 to 5 MΩ. The microelectrode tips were visually positioned in the basolateral region of the amygdala (between the external capsule (EC) and the bed nucleus of the stria terminalis) using a dissecting microscope (see Chapter 4, Fig.1). Field potentials were amplified with a differential amplifier (Warner Instrument Corporation, Hamden, CT, USA). The output was digitized with a

Digidata 1200 interface (Axon Instruments, Foster City, CA, USA). On- and off-line data acquisition and analysis was carried out using the Whole Cell Electrophysiology Program (WCP) version 1.7b (John Dempster, University of Strathclyde, Glasgow, UK).

Stimulation

Synaptic responses were evoked with sharpened tungsten bipolar stimulating electrodes (World Precision Instruments, Sarasota, Florida) placed in the EC (see Chapter 4, Fig.1). The stimulating electrode was ~2 mm from the recording site. Stimuli were delivered using photoelectric stimulus isolation units having a constant current output (ISO-Flex; Stimulus Isolation Unit, Jerusalem, Israel). The stimulus intensity was adjusted to produce a synaptic response 30–50% of maximum amplitude. Single 0.1 ms monophasic square pulses were applied continuously throughout the experiment at 0.1 Hz. Such stimulation was associated with no more than a 10% drift in the peak synaptic response amplitude during experiments lasting up to 120 min.

Drug application

Chemicals and drugs included R-α-methylhistamine dihydrobromide, clonidine and yohimbine were from Tocris Cookson (Ellisville, Missouri). Histamine, norepinephrine, and prozosin were obtained from Sigma (St.Louis, Missouri). Drugs were dissolved in distilled water or Dimethyl sulfoxide (DMSO, Fisher Biotech), neutralized and stored as stock solutions, 1000 times the required concentration, until use. The stock solution was kept for no more than 10 days. During the experiments the drugs were applied to the perfusion solution at desired concentrations.

Data are expressed as mean ± S.E.M. Statistical comparisons were made with Student's unpaired or paired *t*-test, as appropriate.

RESULTS

1. The suppressive effect of the H_3 receptor agonist R-α-methylhistamine on fEPSPs is not affected by stress.

The previous study has shown that histamine could suppress the field potentials recorded in the BLA and this effect was mediated by the histamine H_3 receptor. Thus, in the control animals, R-α-methylhistamine (2 μM), a H_3 receptor agonist, suppressed field potentials to 66.2 ± 9.3% of the baseline values (Figure 1A, B). In stressed animals, R-α-methylhistamine (2 μM) decreased the fEPSPs to 62.4 ± 7.5% of the baseline values, which is not significantly from the control (p>0.05, n=13, Figure 1A, B). These results indicate that the H_3 receptor signaling in the BLA is not changed by stress.

2. The suppressive effect of the α_2 adrenoceptor on fEPSPs is not affected by stress.

The previous study has shown that norepinephrine could suppress excitability of the BLA and this effect is partially mediated by the α_2 adrenoceptor. As illustrated in Figure 2A, norepinephrine (10 μM) suppressed the field potentials in the BLA to 53.2 ± 12.2% of the baseline values (n =12, $p \leq 0.01$). The effect could be blocked by α_2 adrenoceptor antagonist yohimbine, and mimicked by the selective α_2 adrenoceptor agonist clonidine (Figure 2B, C), indicating that the α_2 adrenoceptor mediates norepinephrine-induced suppression of field potentials in the BLA.

In animals sacrificed immediately after stress, clonidine (50 μM) suppressed the fEPSPs to 55.6±6.2% of the baseline values, which was not significantly different from the effect of clonidine in control animals (60.2 ± 5.7% of the baseline values, n =12,

$p>0.05$) (Figure 3A, B). In stressed animals sacrificed 7 days after stress, clonidine (50 μM) suppressed fEPSPs to 60.7 ± 13.9 % of the baseline values, which is not significantly different from the control (57.2 ± 7.3 % of the baseline values) (Figure 3C, 3D). The results indicate that the α_2 adrenoceptor signaling in the BLA is not significantly changed by traumatic stress.

DISCUSSION

The present study indicated that the H_3 receptor and α_2 adrenoceptor-mediated suppression of excitatory synaptic transmission in the BLA was not altered by traumatic stress. The histamine H_3 receptor, at least in the BLA, was not subject to change after stress, indicating that this histamine receptor may not be involved in the pathophysiology of stress. However, it can't be excluded that this receptor in other brain regions may experience changes after stress. Since the primary role of the H_3 receptor is to serve as an autoreceptor in the histaminergic terminals to control the release of histamine (Arrang et al., 1983;Arrang et al., 1985;Brown et al., 2001), the amygdala (the H_3 receptor here is a heteroreceptor regulating glutamate release) may be not a good brain region to study this receptor alteration as a function of stress. However, given that there is no much evidence regarding the involvement of the histaminergic system in pathophysiology of stress-associated psychiatric disorders, this system may not experience much change after stress.

Although alterations in the α_2 adrenoceptor after stress has been repeatedly reported after stress, including in the amygdala, the present study did not demonstrate that this receptor signaling (suppression of glutamate release) experienced a significant change after stress. Three days of stress did tend to increase this receptor-mediated suppression of excitatory synaptic transmission, although not significantly. This implicates that a longer period of stress than the present protocol may be able to induce a significant effect on α_2 adrenoceptors in the amygdala. This may be true given that a previous report used a 21 day-long stress to induce a significant change of this receptor in prefrontal cortex (Flugge, 1996). The negative result of the present study may also be due

to the fact that the stress protocol used in the present study differs from the protocol used in other studies (Nukina et al., 1987).

Another possible reason for our negative result is that the BLA may not be a good brain region to study alteration of the α_2 adrenoceptor as a function of stress. In the amygdala, the α_2 adrenoceptor is a heteroreceptor regulating glutamate release from the glutamatergic terminals, while the primary role of this receptor in the brain is to serve as an autoreceptor, providing a negative feedback to control norepinephrine release from noradrenergic terminals originated in the LC. Therefore, the best brain site for studying α_2 adrenoceptors is the LC, whether α_2 adrenoceptors serve as a "blake" to control the LC firing. Indeed, a previous study, using a similar stress protocol, suggested that this receptor in the LC could be impaired by stress (Simson and Weiss, 1988). Subsensitivity of this receptor in the LC may result in hyperresponsiveness of the noradrenergic system. Actually, it has long been suggested that sensitization of the noradrenergic systems contributes to arousal systems in PTSD including hyperviliance, exaggerated startle, anger and insomnia (Southwick et al., 1999a). Thus, to examine this receptor alteration in the LC as a function of stress we may be able to obtain direct evidence regarding whether the occurrence of the symptoms in the stress-associated diseases is associated with sensitization of the noradrenergic systems.

In summary, BLA H_3 receptors and α_2 adrenoceptor signaling was not altered by stress, indicating that stress-induced alterations in the amygdala aminergic sytems may be specific to 5-HT_{2A} receptors. However, it can't be excluded that H_3 receptors and α_2 adrenoceptors in other brain regions or other monoaminergic receptor subtypes may be

altered by stress. LC may be a better site to examine the functional change of α_2 adrenoceptors after stress.

Figure 1

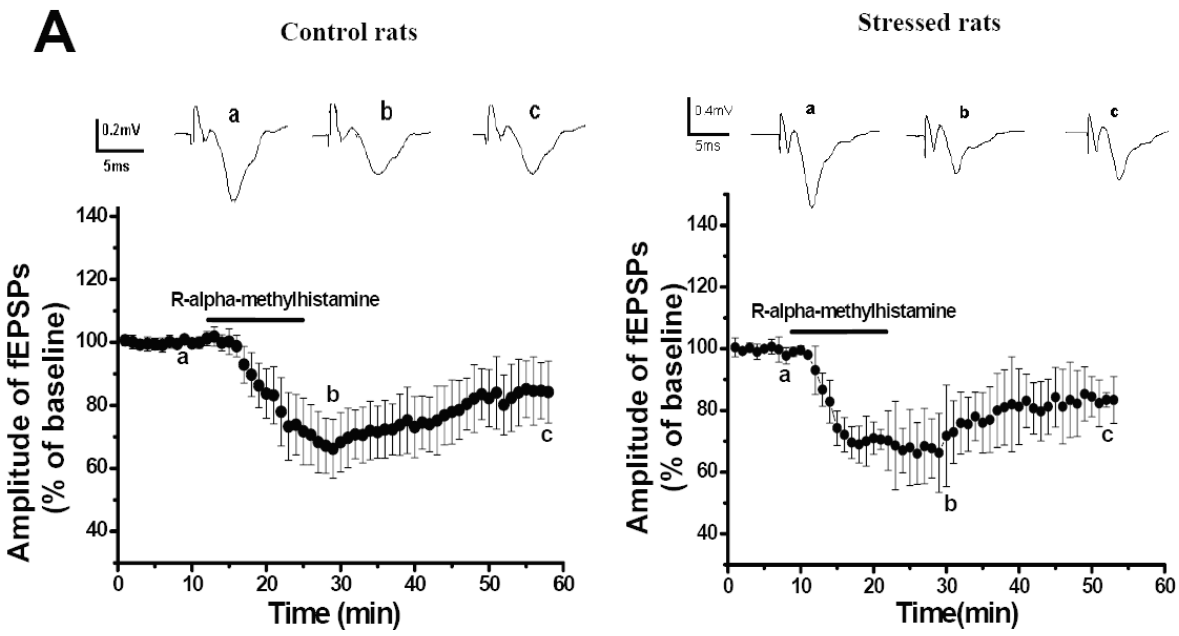


Figure 1. Histamine H_3 receptor-mediated effect is not changed by stress. (**A**)**.** The time course of the effect of R-α-Methylhistamine on field potentials in both control and stressed animals. (**B**). The maximal suppressive effect of R-α-Methylhistamine on field potentials in both control and stressed animals.

Figure 2

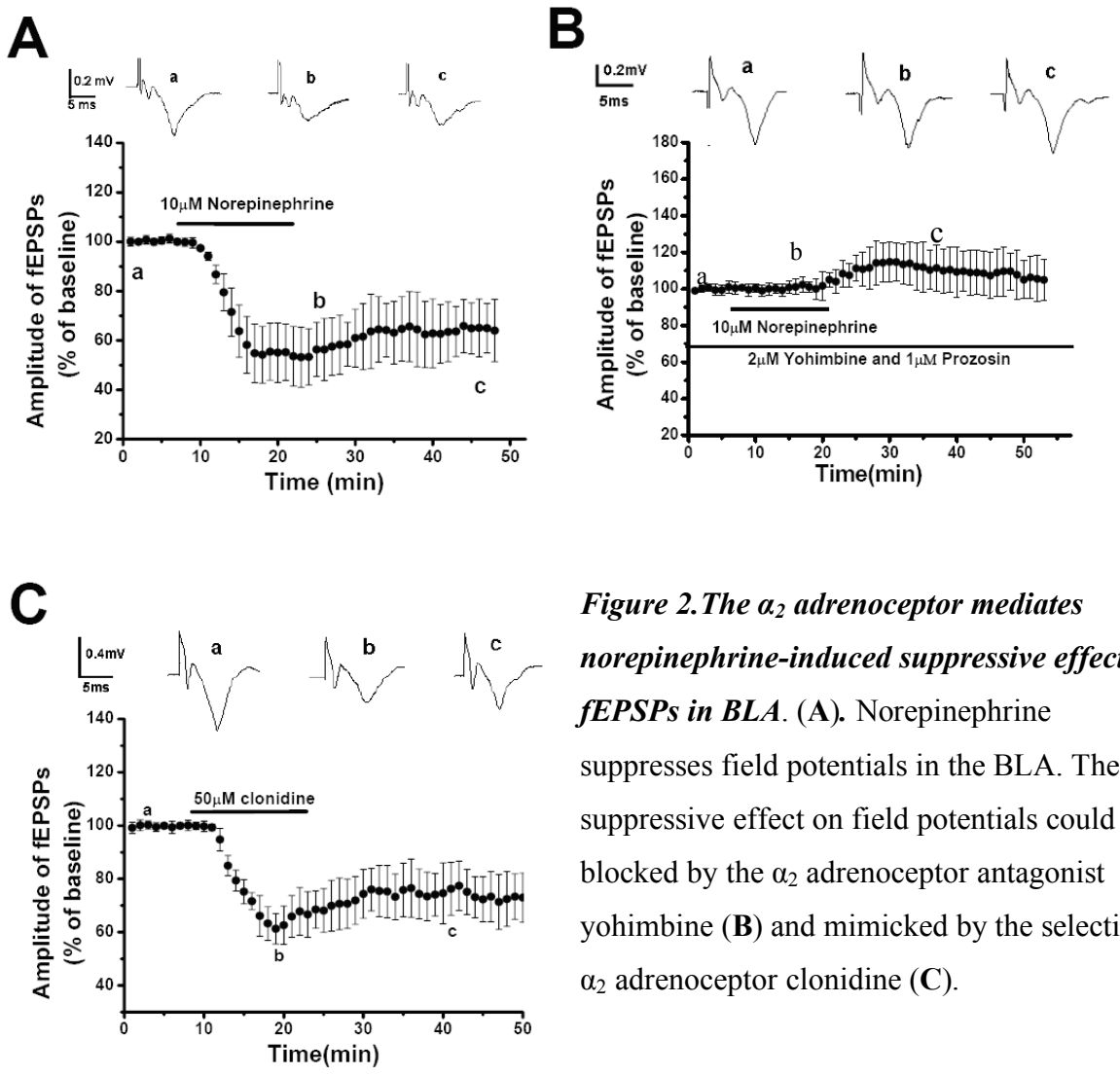
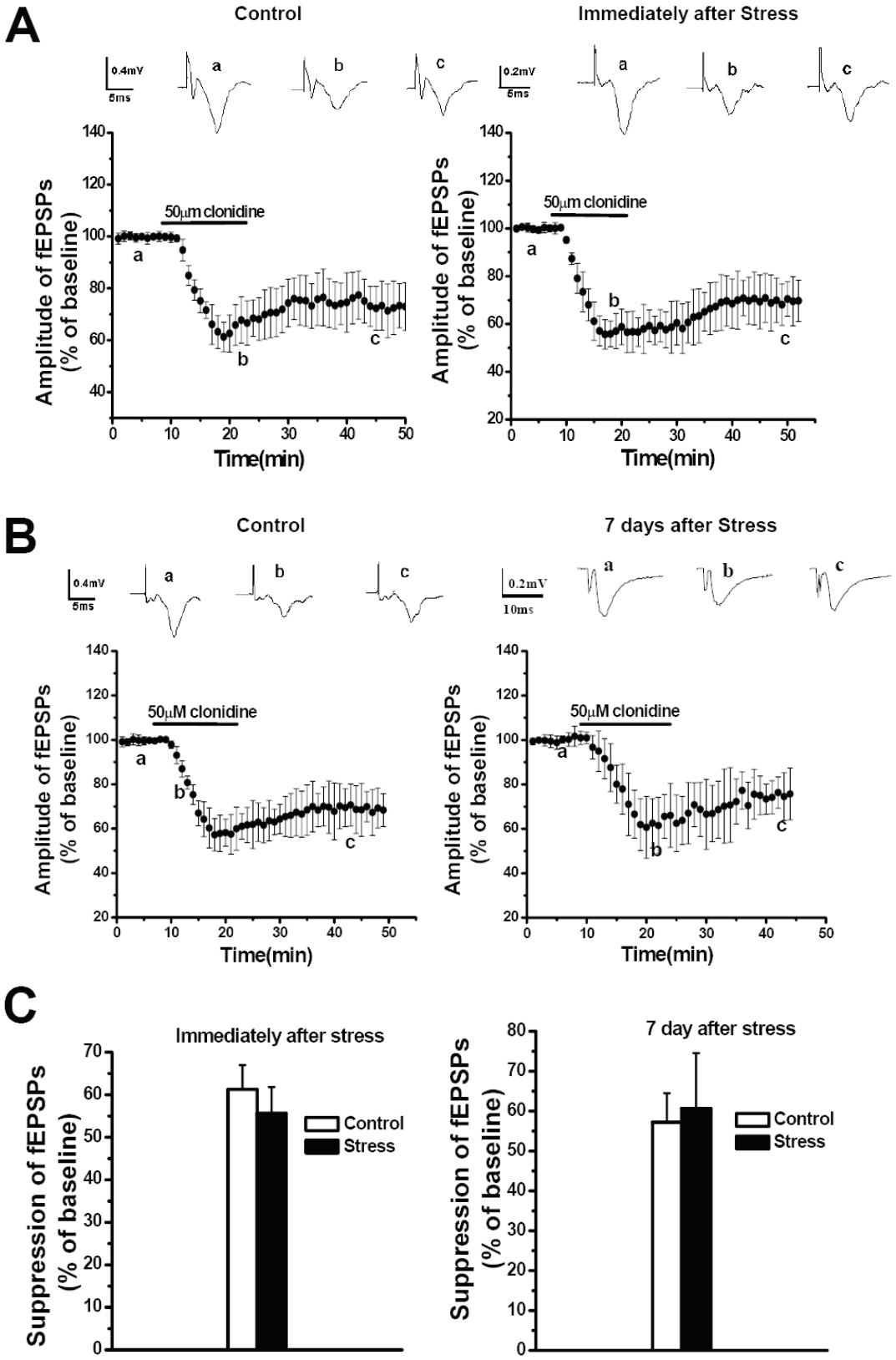


Figure 2.The α_2 adrenoceptor mediates norepinephrine-induced suppressive effect on fEPSPs in BLA. (**A**)**.** Norepinephrine suppresses field potentials in the BLA. The suppressive effect on field potentials could be blocked by the α_2 adrenoceptor antagonist yohimbine (**B**) and mimicked by the selective α_2 adrenoceptor clonidine (**C**).

Figure 3. The α_2 adrenoceptor-mediated suppressive effect in the BLA is not significantly affected by stress. (**A**). The suppressive effect of clonidine on field potentials recorded in the BLA from control animals and the animals sacrificed immediately after stress. (**B**). The suppressive effect of clonidine on field potentials recorded in the BLA in control animals and the animals sacrificed 7 day after stress. (**C**).The maximal suppressive effect of clonidine on field potentials in the BLA in control animals and stressed animals (immediately after stress and 7 days after stress.)

Figure 3


A
Control
Immediately after Stress
0.4mV
5ms
0.2mV
5ms
a
b
c
50μm clonidine
Amplitude of fEPSPs (% of baseline)
Time(min)
B
Control
7 days after Stress
0.4mV
5ms
0.2mV
10ms
50μM clonidine
Amplitude of fEPSPs (% of baseline)
Time(min)
C
Immediately after stress
7 day after stress
Suppression of fEPSPs (% of baseline)
Control
Stress

CHAPTER 6

Discussion

Summary of Findings

5-HT_{2A} receptor is the primary serotonin receptor involved in the serotonergic facilitation of GABA release in the BLA.

Many studies have shown that the primary function of serotonin in the BLA is to facilitate GABA release (Rainnie, 1999;Stein et al., 2000;Stutzmann et al., 1998;Stutzmann and LeDoux, 1999). Although the 5-HT_2 and/or 5-HT_3 receptor have been implicated (Koyama et al., 2002;Morales and Bloom, 1997;Rainnie, 1999;Stein et al., 2000), the exact receptor subtypes involved was still inconclusive. The present study, with multiple approaches, clarified that the 5-HT_{2A} receptor is the primary receptor involved in serotonergic facilitation of GABA release in the BLA.

Since the application of the 5-HT_3 receptor agonist, 2-Methyl-5-HT, in the BLA did not induce a significant change in sIPSCs (data not shown), the 5-HT_3 receptor appears to play a limited role in the serotonergic facilitation of GABA release in the BLA. This result seems to be surprising given that this receptor has been shown to be present in interneurons of the BLA (Morales and Bloom, 1997). As a previous study has shown, the 5-HT_3 receptor may be present in only a subset of GABAergic terminals (Koyama et al., 2002) and very easily desensitized by the bath perfusion of agonists (Belelli et al., 1995), which may explain our negative result.

The present study provided strong evidence showing that the 5-HT_{2A} receptor is the primary receptor responsible for serotonergic facilitation of GABA release in the

BLA. First of all, we found that the 5-HT_2 receptor agonist, α-Methyl-5-HT, mimicked serotonin to facilitate GABA release, while the 5-HT_2 receptor antagonist, Ketanserin, blocked the facilitatory effect. This finding excludes other serotonin receptor subtypes, such as the 5-HT_4 and 5-HT_7 receptors as mediators of GABAergic activity. Since there are three subtypes of 5-HT_2 receptors, that is, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors, we also further identified which 5-HT_2 receptor subtype is involved in this serotonergic facilitatory effect on GABA release. The evidence from the combination of the different antagonists and agonists with different selectivity for these subtypes, indicates that the 5-HT_{2A} receptor is involved (see Chapter 2, Fig.3, 4). This result is consistent with other evidence in the literature. For example, the 5-HT_{2B} receptor is primarily localized to the stomach fundus and has a very restricted expression pattern in the central nervous systems (Leysen, 2004). There is no evidence to show that this receptor is expressed in the basolateral amygdala. 5-HT_{2A} and 5-HT_{2C} receptors have been shown to be expressed in the BLA (Pompeiano et al., 1994;Xu and Pandey, 2000). However, previous studies demonstrated that the activation of 5-HT_{2C} receptors in the BLA appeared to induce anxiety-like effect in animals (Hackler et al., 2006;Campbell and Merchant, 2003;Antonio Pedro de Mello Cruz et al., 2005). These observations actually exclude the 5-HT_{2C} receptor from the receptor candidates involved in the serotonergic facilitation of GABA release, because any mediator facilitating GABAergic synaptic transmission in the BLA should induce an anxiolytic effect as opposed to an anxiogenic effect. Thus, the 5-HT_{2A} receptor subtype appears to be the foremost candidate for the mediation of serotonergic facilitation of GABA release. Indeed, 5-HT_{2A} receptor-mediated facilitation of GABA release in the BLA is also compatible with the evidence from previous

behavioral studies, which have shown that activation of this receptor induced an anxiolytic effect (Ripoll et al., 2006;Bourin et al., 2005;Nic Dhonnchadha et al., 2003). Finally, our immunofluorescence data showed that the 5-HT_{2A} receptor labeling was primarily localized to the soma and dendrites of interneuron-like cells in the BLA, and that the majority of the signal overlapped with the labeling for the interneuron marker, parvalbumin (see Chapter 2, Fig. 6). Interestingly, 5-HT_{2A} receptor immunofluorescence was found to be rarely observed in the pyramidal cells of the BLA, indicating that 5-HT_{2A} receptor expression is restricted to interneurons in the BLA, while the 5-HT_{2C} receptor may be primarily expressed in the pyramidal cells.

The electrophysiological function of histamine in the BLA

Brain histamine is exclusively synthesized by neurons of the tuberomammillary nucleus (TM) of the hypothalamus (Brown et al., 2001;Panula et al., 1989). The nucleus innervates practically the entire brain, with the hypothalamus, basal forebrain, and amygdala receiving a particularly strong innervation in rats (Brown et al., 2001). This innervation is compatible with its role in mediating the response to different types of aversive stimuli (Brown et al., 2001). Although the amygdala receives heavy histaminergic innervation and histamine receptors have been shown to be expressed in the amygdala (Ryu et al., 1995a;Schwartz et al., 1991a), the electrophysiological function of histamine in the amygdala has never been investigated.

The present study, for the first time, shows that the primary function of histamine in the BLA is to suppress excitability of BLA neurons, especially when applied at a high concentration. This effect is mediated by the H_3 receptor, which is localized on the glutaminergic terminals of the BLA. Activation of this receptor could decrease glutamate

release, thereby reducing the glutaminergic synaptic transmission in the BLA. Indeed, many studies, especially amygdala-kindling studies, have suggested that histamine exerts an inhibitory effect on amygdala excitability (Kamei, 2001a;Kamei, 2001b;Ishizawa et al., 2000). However, the mechanisms underlying these effects are not clear. The present study, utilizing the condition-controlled slice system, clarified an important mechanism underlying histamine-induced antiepileptic effects observed in many amygdala-kindling studies. The histamine H_3 receptors may be the most important mediator involved in the histamine-induced antiepileptic effects in the amygdala, the histamine-induced inhibition of conditioned avoidance response and context conditioning (Tasaka et al., 1985;Alvarez and Ruarte, 2002).

A more interesting and novel finding was that histamine, at relatively low concentrations, potentiated excitatory synaptic transmission in certain BLA neurons, and this effect was not associated with currently known histamine receptors. This suggests the existence of a novel unidentified histamine receptor in the brain. To date, there are at least three identified histamine receptors at the CNS, H_1, H_2 and H_3 (Brown et al., 2001). The present work does not support the involvement of any histamine receptor types in the histamine-induced potentiation of excitatory synaptic transmission. Recently, a novel H_4 receptor has been identified, but there is no evidence that this receptor is expressed in the CNS. In addition, our pharmacological data do not support the idea that an H_4 receptor is involved in this effect (see Discussion in Chapter 4).

In fact, several observations by other investigators suggest that there are likely additional histamine receptor subtypes. In cholinergic neurons of the medial septum, Gorelova and Reiner (Gorelova and Reiner, 1996) have described a slow depolarisation

elicited by histamine which is elicited by a decrease in an inwardly rectifying potassium channel. This depolarization was not blocked by antagonists of histamine H_1 or H_2 receptors and was not mimicked by an agonist of the histamine H_3 receptor. Yang and Hatton (Yang and Hatton, 1994) observed short-latency fast IPSPs in rat supraoptic oxytocin neurons following stimulation of the tuberomamillary nucleus (TMN) which were not blocked by the selective $GABA_A$ receptor antagonist, bicuculline, but was blocked by the chloride-channel blocker, picrotoxin, and the histamine H_2 receptor antagonists, cimetidine and famotidine. Thus, histamine in the rodent brain appears to be able to activate a chloride channel, as found in the invertebrate. Finally, histamine has been observed to prime adenosine modulation of excitability, which is also not related to any currently known histamine receptors (Brown et al., 2001). These observations, together with our data, suggest that our knowledge of the histamine receptor family may be far from complete.

5-HT_{2A} receptor and stress-enhanced ASR.

The serotonergic system in the brain has long been recognized for its role in the stress response, and dysregulation of this system has been closely linked with multiple anxiety and stress disorders (Eison and Eison, 1994). With seven distinct receptors, and multiple subtypes, the serotonin receptor system is perhaps the most complex aminergic system in the brain (Saxena, 1995). For response to stress, the 5-HT_{2A} receptor appears to be key. On the one hand, this receptor participates in the stress response by stimulating the release of CRF from the paraventricular nucleus (Zhang et al., 2002;Mikkelsen et al., 2004;Hanley and Van de Kar, 2003;Van de Kar et al., 2001;Saphier et al., 1995). On the

other hand, the 5-HT_{2A} receptor is readily altered by different types of stressful experiences, while other serotonin receptors appear to be more resistant to modification by stress. For example, 5-HT_{2A} receptor-mediated behaviors are altered by footshock and swim stress (Izumi et al., 2002;Pericic, 2003), and inescapable stress induces a decrease in 5-HT_{2A} receptor levels in the hippocampus (Dwivedi et al., 2005;Wu et al., 1999), amygdala (Wu et al., 1999), hypothalamus (Dwivedi et al., 2005;Petty et al., 1997;Wu et al., 1999), while simultaneously increasing levels in the prefrontal cortex (Dwivedi et al., 2005). Alteration of this receptor, then, appears to significantly contribute to the occurrence of the symptoms associated with anxiety and stress disorders. If this is true, a question follows: How could alteration of this receptor contribute to the occurrence of these symptoms?

The present finding suggests there may be a relationship between alterations in 5-HT_{2A} receptor signaling and known behavioral consequences of stress. Specifically, alteration in 5-HT_{2A} signaling in the basolateral amygdala may be associated with stress-induced enhanced ASR (Sananes and Davis, 1992;Van Nobelen and Kokkinidis, 2006).

Enhanced ASR has enjoyed considerable face validity as a behavioral manifestation of fear in animals. It is widely used as a reliable nonverbal index of fear and anxiety in human experimental subjects after the presentation of aversive stimuli (Bradley et al., 2001). Although it is still debated whether enhanced ASR after the presentation of restraint/tail shock represents the unconditioned fear acquired after the aversive stimuli or conditioned fear response to context, this enhanced ASR depends on the amygdala (Hitchcock et al., 1989;Sananes and Davis, 1992;Adamec et al., 1999;Gewirtz et al., 1998;Davis et al., 1997b;Davis et al., 1997c;Van Nobelen and

Kokkinidis, 2006). Certain studies also suggested that the decreased GABAergic transmission in the BLA may be responsible for the enhanced ASR (Van Nobelen and Kokkinidis, 2006;Stork et al., 2002). Consistent with this, our results demonstrated the 5-HT_{2A} receptor-mediated tonic facilitation of GABA release was severely impaired after exposure to stress, and that this impairment may be an important mechanism underlying enhanced ASR.

Our behavioral data also support this contention. Administration of the 5-HT_{2A} receptor antagonist during stress is assumed to prevent this receptor from being impaired. Thus, this treatment should maintain 5-HT_{2A}-receptor-mediated facilitation of GABA release intact, and enhanced ASR should be prevented. Our data indeed demonstrated that after the treatment with the antagonist, enhanced ASR was prevented in these stressed animals, which otherwise experience long-lasting enhanced ASR after stress. The data suggest that there is a causal relationship between impaired 5-HT_{2A} receptor signaling in the BLA and enhanced ASR.

As stated in the Introduction, the amygdala's response to aversive stimuli reflects a common, multimodal feature of amygdala coding, and this coding requires an intact serotonergic transmission. In the normal amygdala, basal levels of serotonin, acting via the 5-HT_{2A} receptor, may contribute to tonic inhibition of BLA pyramidal neurons (Stutzmann et al., 1998;Stutzmann and LeDoux, 1999), which allows glutamatergic sensory input to be more effectively filtered in order to permit relevant stimuli to be fully processed by the amygdala. Decreased serotonergic functioning might result in deficient GABAergic modulation of excitatory sensory afferents, which perhaps allows innocuous sensory signals to be processed in the amygdala and activate downstream responses

initiated by the amygdala (Figure 1). Enhanced ASR is an important downstream response initiated by activation of the amygdala (Koch and Schnitzler, 1997)(Figure 1).

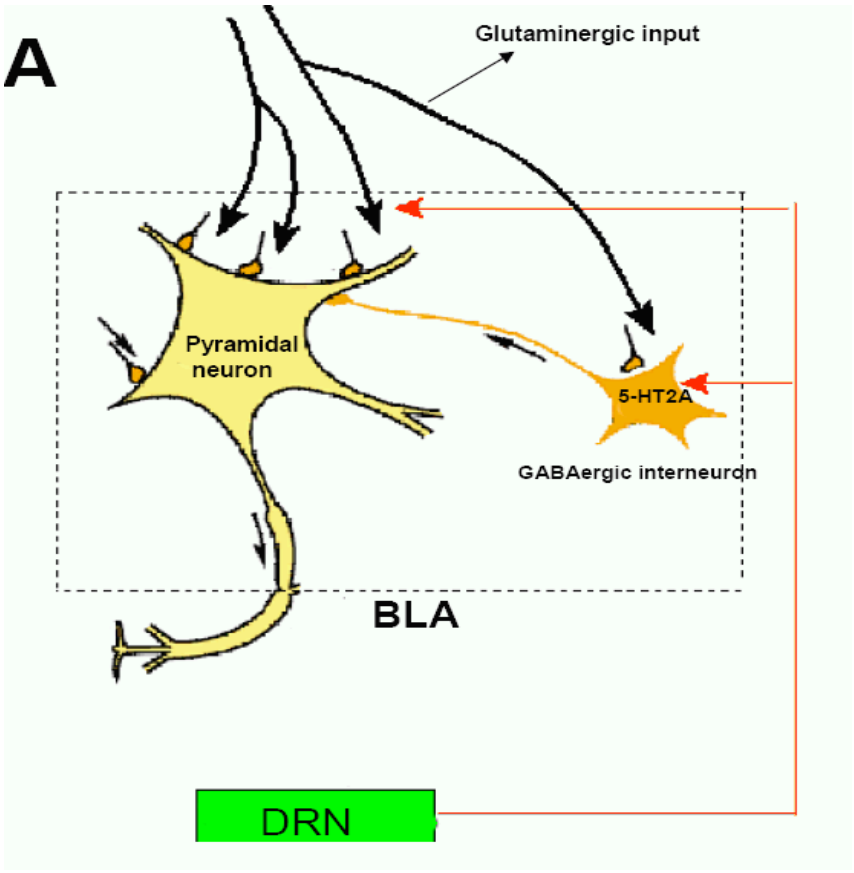
5-HT_{2A} and stress-induced sustained weight loss

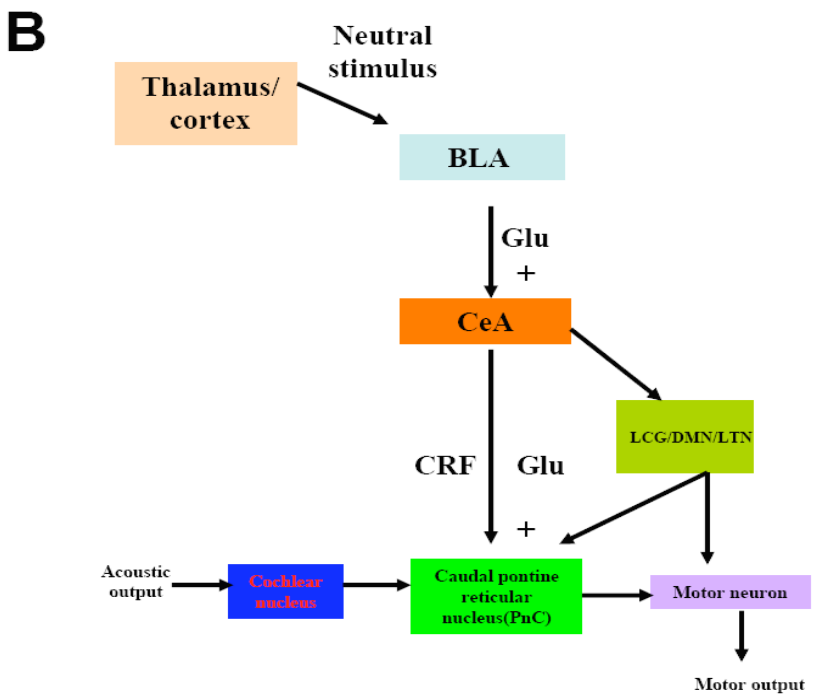
Sustained body weight loss has been long regarded as a prominent symptom in certain patients with depression and anxiety disorders (Hirschfeld et al., 2005;Evers and Marin, 2002;Hopkinson, 1981). This includes children with anxiety and stress disorder experiences, who can sustain a retardation in body growth (Richards et al., 2006;Yorbik et al., 2004). It is well established that the serotonergic system is an important mechanism regulating energy feeding and body weight (Heisler et al., 2003;Simansky, 1996), and dysregulation of serotonin function in the CNS contributes to core symptoms in patients with eating disorders (Wolfe et al., 1997). Thus, the weight loss associated with anxiety and stress disorders may partially be due to dysregulation of the serotonergic system (Wolfe et al., 1997;Franke et al., 2003;Hassanyeh and Marshall, 1991).

The hypothalamic paraventricular nucleus (PVN) has been proposed as an important terminal field that is involved in 5-HT's role in appetite control, although recent studies implicate extra-PVN regions in this function**.** In the hypothalamus, 5-HT_{1B} and 5-HT_{2C} receptors appear to be the primary serotonin receptors involved in serotonergic regulation of energy balance (De Vry and Schreiber, 2000). Activation of the hypothalamic 5-HT_{2C} receptor during stress is believed to contribute to stress-induced reductions of food intake (Grignaschi et al., 1993), while activation of hypothalamic 5-HT_{2A} receptors do not affect single feeding bouts. However, the level of the hypothalamic5-HT_{2A} receptors appears to be positively proportional to body mass (see Chapter 4 Discussion),

Figure 1. The proposed mechanism by which malfunctioning of the 5-HT_{2A} receptor in the basolateral amygdala (BLA) contributes to enhanced acoustic startle response (ASR). The BLA receives heavy serotonergic innervation from the dorsal raphe nucleus (DRN) illustrated by red arrows. The BLA projecting neuron is under inhibitory control by tonic serotonergic activation of GABAergic interneurons mediated by 5-HT_{2A} receptors (**A**). (**B**). Malfunctioning of this receptor allows innocuous stimuli, which otherwise would be filtered, to activate the projecting neurons. These activated neurons project to the pontine reticular nucleus (PnC) to potentiate the ASR (Koch and Schnitzler, 1997).

Figure 1


A
Glutaminergic input
Pyramidal neuron
5-HT2A
GABAergic interneuron
BLA
DRN


B
Neutral stimulus
Thalamus/ cortex
BLA
Glu
+
CeA
LCG/DMN/LTN
CRF
Glu
+
Acoustic output
Cochlear nucleus
Caudal pontine reticular nucleus(PnC)
Motor neuron
Motor output

suggesting that this receptor in the hypothalamus may be involved in the regulation of long-term energy balance. Interestingly, this hypothalamic receptor is subject to change after stress. Different types of stress could decrease this receptor in the hypothalamus, while 5-HT_{2C} receptors remain unchanged (Dwivedi et al., 2005;Petty et al., 1997;Wu et al., 1999). This raised the possibility that dysregulation of the serotonergic system in the hypothalamus, resulting from the alteration of 5-HT_{2A} receptors, may participate in the occurrence of sustained weight loss observed in stressed animals.

Compatible with previous reports, our data did show that hypothalamic levels of 5-HT_{2A} receptor mRNA was decreased by stress, while 5-HT_{2C} receptor mRNA remained unchanged. More interestingly, pretreatment with MDL11,939 completely reversed the sustained weight loss of stressed animals.

As stated in the Discussion of Chapter 3, hypothalamic 5-HT_{2A} receptor levels appear to determine the individual's final body weight. Increased levels of hypothalamic 5-HT_{2A} receptors may induce obesity, while decreased levels may induce a low body weight. Stress-induced decreases in the hypothalamic 5-HT_{2A} receptor may, thus, determine the sustained body weight loss in stressed animals. Since the hypothalamic 5-HT_{2A} receptor downregulation should result from prolonged stimulation by stress-elevated 5-HT, and antagonists can prevent agonist-induced downregulation of receptor gene expression (Brown et al., 1998;Bieck et al., 1992;Baumhaker et al., 1993;Horackova et al., 1990;Lassegue et al., 1995;Scarceriaux et al., 1996;Chau et al., 1994;Fukamauchi et al., 1993), MDL 11,939 would prevent decreased expression of this receptor. A normal level of the hypothalamic 5-HT_{2A} receptor in animals pretreated with MDL

11,939 may allow the animal to detect abnormal body weight and to overeat to achieve normal body weight.

In adult individuals, body weight is maintained at a relatively stable level for long periods. A set-point theory has been proposed to explain long-term maintenance of body weight (Harris, 1990). This theory suggests that body weight is regulated at a predetermined, or preferred, level by a feedback control mechanism. Information from the periphery is carried by an affector to a central controller located in the hypothalamus. The controller integrates and transduces the information into an effector signal that modulates food intake or energy expenditure to correct any deviations in body weight from set-point. The observation that the level of hypothalamus 5-HT_{2A} receptors seems to be positively proportional to body mass, that genetic variations of the 5-HT_{2A} receptor can induce abdominal obesity in humans (Rosmond et al., 2002b;Rosmond et al., 2002a), and the present findings, suggest that the hypothalamic 5-HT_{2A} receptor may be an important component of "set-point" of body weight. The high or low level of the hypothalamic 5-HT_{2A} receptor expression may determine set-point, and thus determine an individual's final body weight.

Role of the 5-HT_{2A} receptor in pathophysiology of stress and stress-induced anxiety and depressive disorders.

In fact, sustained weight loss and enhanced ASR may represent behavioral and physiological manifestations of sustained anxiety status or depression. The findings that stress impaired 5-HT_{2A}-mediated facilitation of GABA release in the amygdala, that stress induces a decrease of this receptor in the hypothalamus, and that pretreatment with

a 5-HT_{2A} receptor antagonist could prevent the development of behavioral and physiological manifestations of anxiety status, suggest that altered 5-HT_{2A} receptor signaling is closely associated with the occurrence of anxiety and stress disorders.

The inescapable stress protocol we employed has been used in many studies to try to understand the role of the serotonergic system in the pathogenesis of depression and anxiety-like symptoms. It is recognized that such stress could induce sustained activation of the DRN-corticolimbic pathway. This continuous activation of the DRN, and sustained high level of serotonin in the amygdala, hippocampus, prefrontal cortex, hypothalamus has been shown by many studies to be responsible for the occurrence of pathophysiological change associated with multiple anxiety and stress disorders (Maier and Watkins, 2005;Minor and Hunter, 2002). However, the mechanism by which such continuous activation of this system transforms the animals to the pathophysiological state is not clear.

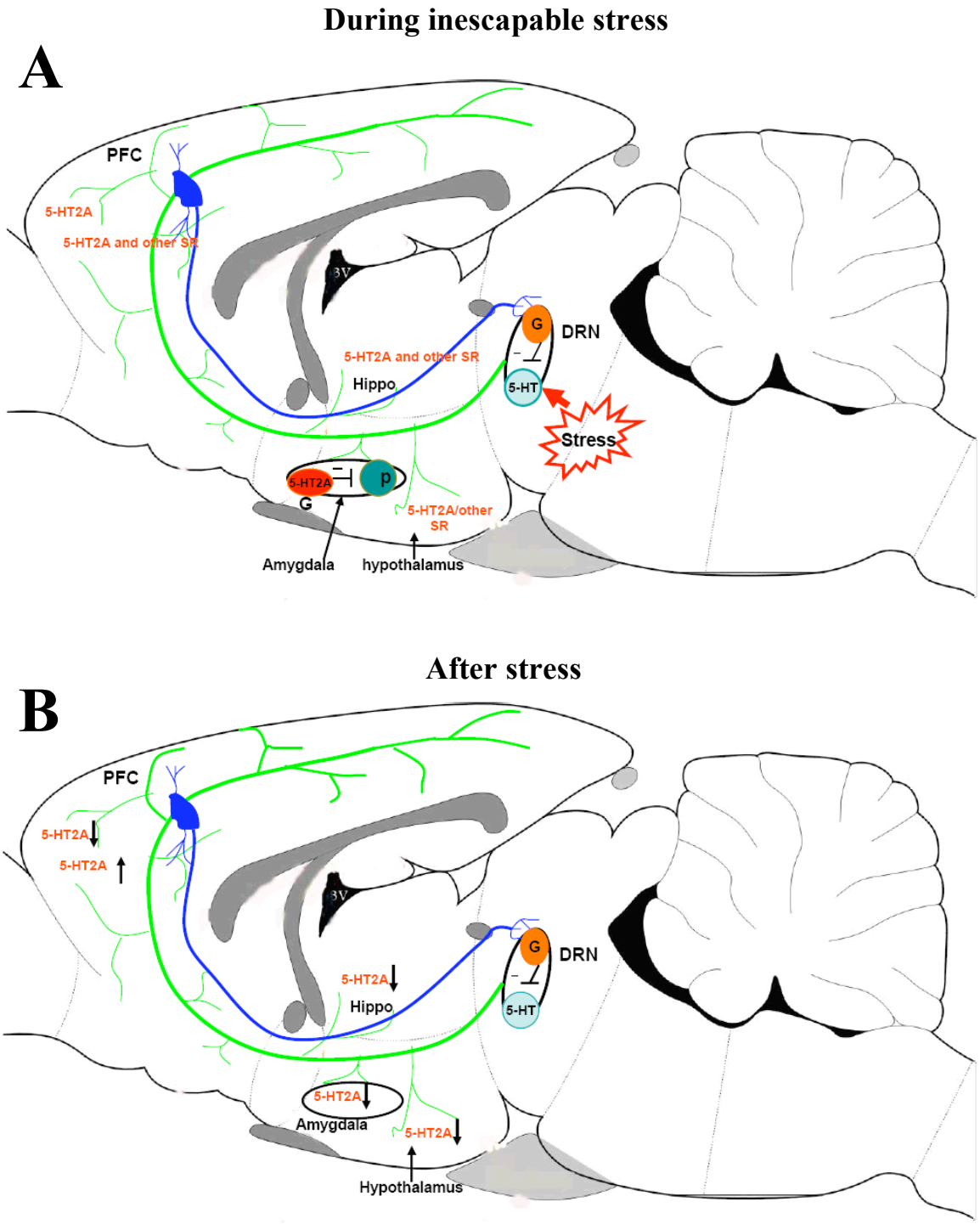
The present study may unravel an important mechanism by which such sustained activation of this system in corticolimbic structures could transform the normal brain to the "anxious" brain or the "depressive" brain. As shown by the data presented, as a consequence of such sustained high levels of serotonin, the 5-HT_{2A} receptor in the BLA was downregulated and desensitized. Such sustained enhanced serotonin could also induce a decrease of 5-HT_{2A} receptor expression in the hippocampus (Dwivedi et al., 2005), and hypothalamus (Dwivedi et al., 2005;Wu et al., 1999), and decreases or increases this receptor in the prefrontal cortex (Dwivedi et al., 2005;Harvey et al., 2003). In fact, 5-HT_{2A} receptors mediate tonic, serotonergic facilitation of GABAergic neurons not only in the amygdala, but also in other corticolimbic structure including the

hippocampus (Shen and Andrade, 1998), prefrontal cortex and other cortical areas (Marek and Aghajanian, 1994;Minor and Hunter, 2002;Xu and Pandey, 2000;Cozzi and Nichols, 1996;Abi-Saab et al., 1999;Zhou and Hablitz, 1999;Jakab and Goldman-Rakic, 2000). Downregulation of the 5-HT_{2A} receptor in these corticolimbic structures, especially in the amygdala, will result in a severe impairment of tonic facilitation of GABA release mediated by the 5-HT_{2A} receptor. As a consequence of reduced GABA tone in the corticolimbic structures, a sustained anxious status ensues (Millan, 2003)(see Figure 2).

As many studies have indicated, activation of the DRN-corticollimbic pathway in response to potential or distal threat stress facilitates active escape or avoidance behaviors (Graeff et al., 1996). Thus, this pathway is an important component of the central network that protects the organism from being harmed. This process may require activation of postsynaptic 5-HT_{2A} receptors in the amygdala and prefrontal cortex (Carli et al., 2006;Mirjana et al., 2004;Pehek et al., 2006;Williams et al., 2002;Wu et al., 1999). Thus, dysregulation of this receptor in these structures by inescapable stress may be responsible for impairment of avoidance behavior (behavioral depression) developed in these animals. However, whether or not protection of this receptor with the 5-HT_{2A} receptor antagonists during stress could prevent the development of such behavioral depression has never been examined before (see Figure 2).

Figure 2. The proposed mechanisms by which dysregulation of serotonergic systems in its diverse terminal domains contributes to anxiety status. (**A**).Stressors activate the DRN-corticolimbic pathway, and increase serotonin levels in the amygdala, prefrontal cortex (PFC) and other structures. This facilitates active escape or avoidance behaviors so that the animals could protect themselves from being harmed. Such behaviors may require the coordination of the amygdala and PFC and depend on activation of certain postsynaptic serotonin receptor, such as the 5-HT_{2A} receptor in these structures. When the stressor is avoided and removed, the DRN activity returns to the resting status or is deactivated by inhibitory control from the PFC. Stressors, if inescapable, also activate the DRN-corticolimbic pathway to allow animals to try to escape or avoid the stressors. However, the escape or avoidance behavior could not be achieved in this situation and stressors are continuously present. As a result, activation of the DRN-corticolimbic pathway will be sustained. One potential consequence of such sustained activation is dysregulation of the DRN-corticolimbic pathway, which may involve both dorsal raphe per se (Maier and Watkins, 2005) and its postsynaptic receptors (**B**). One important receptor is the 5-HT_{2A} receptor. Activation of this receptor in the amygdala and prefrontal cortex during stress, by increasing GABAergic tone and/or other mechanisms, may be critical for the animal to initiate protective behaviors in response to stressors. Impairment of this receptor signaling may result in loss of facilitation of GABAergic tone in corticolimbic structures, especially in the amygdala, and enhanced anxiety status may ensue. Alteration of this receptor signaling in the PFC and amygdala may also be responsible for impairment of avoidance behaviors as a consequence of such stress.

Figure 2


During inescapable stress
A
PFC
5-HT2A
5-HT2A and other SR
3V
G
DRN
5-HT2A and other SR
Hippo
5-HT
Stress
5-HT2A
p
G
5-HT2A/other SR
Amygdala
hypothalamus
After stress
B
PFC
5-HT2A
5-HT2A
3V
G
DRN
5-HT2A
Hippo
5-HT
5-HT2A
Amygdala
5-HT2A
Hypothalamus

Contribution of Present Findings

Converging evidence from a variety of disciplines indicates that pathophysiological alterations in neuronal excitability in the amygdala are characteristic features of certain stress-related psychiatric illnesses, such as PTSD and depressive disorders (Drevets, 2000;Kalia, 2005;Manji et al., 2001;Shin et al., 2006). Neuroimaging studies have consistently shown that amygdala activity to both traumatic reminders and more generally threatening stimuli is exaggerated in PTSD patients. PET scan studies have revealed multiple abnormalities of regional blood flow (RBF) and glucose metabolism in resting, unmedicated patients, with RBF and metabolism found consistently increased in the amygdala (Manji et al., 2001). The present finding may reveal an important pathophysiological mechanism contributing to such hyperactivity in the amygdala in these disorders. Dysregulation of the serotonergic system in these disorders has been recognized for over 40 years. Such dysregulation of this system in the amygdala, specifically, malfunctioning of 5-HT_{2A} receptor-mediated tonic facilitation of GABA release, may result in reduced GABAergic tone and a low intrinsic threshold of amygdala circuitry in response to the sensory input (Villarreal and King, 2001;Drevets, 2003). The consequence of such a change will be hyperresponse of amygdala to traumatic reminder and even innocuous stimuli, which may underlie the symptoms of these disorders.

The finding that MDL 11,939 treatment could prevent the development of behavioral and physiological changes associated with PTSD and anxiety disorders indicates that 5-HT_{2A} receptor antagonists are very promising preventive agents in fighting against these stress-associated disorders, especially PTSD. Indeed, several

relatively selective 5-HT_{2A} antagonists have been recently developed (Bartoszyk et al., 2003;Dudley MW, 1988) and have been in clinical trials for treatments of schizophrenia and insomnia (de Paulis, 2001;Fish et al., 2005). These drugs appear to be well tolerated by all study participants (David et al., 2004). Therefore, clinical trials for PTSD with these compounds should be conducted in the near future. In addition, the findings that both α_2 adrenoceptors and histamine H_3 receptors suppress excitability of the amygdala and they are not altered by stress, suggest that agonists for these receptors may hold promise as treatments for disorders such as PTSD and depression.

Pharmacological prevention and treatment of stress-related mood disorders such as PTSD is a topic of current medical interest that has received limited attention by clinical and neurobiological investigations (Pitman et al., 2002). Most of the research performed thus far has focused on the effectiveness of a few classes of compounds in alleviating the symptoms of stress-related disorders (Albucher and Liberzon, 2002). These include tricyclic antidepressants, monoamine oxidase inhibitors, and serotonin reuptake inhibitors. Although clinical research has shown that these agents are useful in alleviating symptoms and facilitating recovery, their overall efficacy is limited and very often hindered by their serious side effects. The development of more specific pharmacological agents with potentially less significant side effects as a therapeutic strategy aiming at preventing the establishment of stress-related disorders is an important step in the treatment of these illnesses. The present findings reveal that 5-HT_{2A} receptor ligands may be more efficacious pharmacological interventions aimed at regulating neuronal excitability and neuroplasticity in the amygdala, and thus preventing or treating stress-induced affective disorders such as PTSD. Active duty military, reservists and

veterans, as well as civilians exposed to traumatic circumstances, may all benefit from the knowledge thus obtained.

Future Experiments

The present study shows that 5-HT_{2A} receptor signaling in the amygdala is impaired by inescapable stress. As mentioned above, to better appreciate the role of the 5-HT_{2A} receptor in the development of anxiety and stress disorders, alterations of this receptor signaling in other critical brain regions, such as the prefrontal cortex, may also be needed to be examined. In fact, qPCR studies are presently underway in the lab.

The present data are derived from animals immediately after stress or 1 day after stress. In order to determine whether impaired 5-HT_{2A} receptor signaling is a pathophysiological mechanism of anxiety and stress disorders, long-term changes should also be assessed. The preliminary study has shown that 10 days after stress, this receptor still remained downregulated.

As stated in Chapter 2, the reduction of 5-HT_{2A} receptor levels has been repeatedly reported in depressive disorders. In order to better appreciate the role of this receptor change in the pathophysiology of depressive disorders, the association between the behavioral depression developed in this animal model and 5-$HT2_A$ receptors should also be examined.

This study implies that impaired 5-HT_{2A} receptor signaling may be an important pathophysiological mechanism of PTSD. In order to further examine the role of 5-HT_{2A} receptors in this disorder, investigating alterations in this receptor using imaging studies and analyses of human postmortem brains should also be conducted.

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